QTL for white spot syndrome virus resistance and the sex-determining locus in the Indian black tiger shrimp (Penaeus monodon)

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Abstract

Background: Shrimp culture is a fast growing aquaculture sector, but in recent years there has been a shift away from tiger shrimp Penaeus monodon to other species. This is largely due to the susceptibility of P. monodon to white spot syndrome virus disease (Whispovirus sp.) which has impacted production around the world. As female penaeid shrimp grow more rapidly than males, mono-sex production would be advantageous, however little is known about genes controlling or markers associated with sex determination in shrimp. In this study, a mapped set of 3959 transcribed single nucleotide polymorphisms were used to scan the P. monodon genome for loci associated with resistance to white-spot syndrome virus and sex in seven full-sibling tiger shrimp families challenged with white spot syndrome virus.

Results: Linkage groups 2, 3, 5, 6, 17, 18, 19, 22, 27 and 43 were found to contain quantitative trait loci significantly associated with hours of survival after white spot syndrome virus infection (P < 0.05 after Bonferroni correction). Nine QTL were significantly associated with hours of survival. Of the SNPs mapping to these and other regions with suggestive associations, many were found to occur in transcripts showing homology to genes with putative immune functions of interest, including genes affecting the action of the ubiquitin-proteasome pathway, lymphocyte-cell function, heat shock proteins, the TOLL pathway, protein kinase signal transduction pathways, mRNA binding proteins, lectins and genes affecting the development and differentiation of the immune system (eg. RUNT protein 1A). Several SNPs significantly associated with sex were mapped to linkage group 30, the strongest associations (P < 0.001 after Bonferroni correction) for 3 SNPs located in a 0.8 cM stretch between positions 43.5 and 44.3 cM where the feminisation gene (FEM-1, affecting sexual differentiation in Caenorhabditis elegans) mapped.

Conclusions: The markers for disease resistance and sexual differentiation identified by this study could be useful for marker assisted selection to improve resistance to WSSV and for identifying homogametic female individuals for mono-sex (all female) production. The genes with putative functions affecting immunity and sexual differentiation that were found to closely map to these loci provide leads about the mechanisms affecting these important economic traits in shrimp.

Keywords: Penaeus monodon, Single nucleotide polymorphism, White spot syndrome virus, Disease resistance, Sex-linked gene markers, Quantitative trait loci

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Background

Crustaceans make up around 10% of the world’s aquaculture production with average growth in production of 15% per year from 1970 reaching 5 million tonnes in 2008 [1]. Rapid growth during the period 2001–2008 was due to increased production of Litopenaeus vannamei in China, Thailand, and Indonesia. Production of P. chinensis has been reduced, and no significant change in the production of P. monodon has occurred over the last 13 years, mainly because of difficulties due to disease with white spot syndrome virus in these species and the increased availability of genetically improved specific pathogen free L. vannamei post-larvae. More than 80% of shrimp exports from India are derived from aquaculture production.

One of the major worldwide problems limiting the culture of shrimp is viral disease. White spot syndrome virus (family Nimaviridae, genus Whispovirus, WSSV) is a lethal pathogen that can cause up to 100% mortality within 7–10 days on shrimp farms, and has devastated shrimp farming industries across the world (reviews by: [2-4]). Selective breeding has been suggested by many as a highly effective long term strategy to combat the threat of disease [5]. However, resistance to WSSV has low heritability in L. vannamei [6-11], and limited evidence has been found for genetic variation in resistance to WSSV in P. monodon [12,13], especially because of the difficulty with developing a standardized challenge protocol for WSSV. Shrimp exposed to WSSV have a rapid mortality rate and cannibalism can cause secondary waves of infection. Oral infection of individual shrimp with a controlled dose of the virus, although technically difficult and labour intensive, is recommended [8]. Where genetic resistance has been detected, it has been found to be strongly negatively correlated with growth rate [10].

Shrimp have a very limited adaptive immune response [14] and lack diverse immune related molecules such as immunoglobulin, T cell receptor and major histocompatibility complex. The innate immune response of shrimp has been shown to be triggered almost instantaneously in response to peptidoglycan stimulation [15] and is believed to be the primary defence mechanism against infection in this group of species. A number of potential antimicrobial peptide coding genes have been isolated from penaeid shrimp and some such as penaeidins and crustins have been found to be differentially expressed over the time course of infection [16-18]. The susceptibility of P. monodon to white spot disease has been shown to increase when penaeidin class 5 expression is suppressed by interference mediated gene silencing [19]. Shrimp surviving 84 hours post-infection have higher expression of lysozyme, C-type lectin, penaeidins, prophenoloxidase-1 and prophenoloxidase-2 in haemocytes than those dying less than 60 hours post infection [18]. Heat shock protein 21 is down regulated after infection to WSSV [20]. Shrimp lysozyme has been shown to be effective in blocking infection by WSSV in blue shrimp (Litopenaeus stylirostris) [21].

As yet there are no vaccines or other treatments available with proven efficacy against WSSV, although a number of studies have revealed promising leads. The WSSV binding proteins isolated from viral particles in the haemolymph of shrimp infected with WSSV, have been shown to inhibit the binding of this virus to haemolymph cells and improve survival of shrimp [22]. Injection of shrimp with recombinant fortilin after infection with WSSV, results in 80–100% survival and low levels of WSSV are detected, suggesting that fortilin inhibits viral replication [23]. Fortilin is highly upregulated in haemolymph during the early phase of white spot infection [24]. Injection with recombinant ferritin or lysozyme also results in protection to challenge with WSSV [21,25]. Inoculation in feed with bacterially expressed double stranded RNA VP28 (encoding for an envelope protein found in WSSV) and vaccination with VP28 and recombinant VP292 [26-29], as well as exposure to probiotics and beta-1,3/1,6-glucans [30], have been shown to provide improved survivability. Shrimp immunity to WSSV was shown to be enhanced by intramuscular injection of oligodeoxynucleotides with Cytosine-Guanine motifs and Vibrio harveyi DNA [31]. In addition, double stranded RNA of any type has been found to induce antiviral protection in shrimp [32]. Interestingly, a gene designated as PmAV was isolated using differential display from viral resistant shrimp and was shown to have antiviral activity [33].

Resistance to WSSV is a strong candidate trait for marker-assisted or genomic selection since it appears to have low heritability and has a negative correlation with another selected trait (growth). The lack of reported quantitative trait loci associated with this trait may not be due to the lack of segregating genes for resistance, but could instead be due to the highly virulent nature of WSSV, challenge testing methods that do not deliver accurate resistant phenotypes and because marker resources do not sufficiently cover the genome.

Another important factor in shrimp cultivation is sex determination. Female penaeid shrimps grow more rapidly than males and so mono-sex production of females would be advantageous for production [34]. This could also be used to provide a level of genetic protection, hindering the replication of genetically superior stock. In penaeid shrimps, females are known to be heterogametic with sex determined by a WZ-ZZ chromosomal system [35-37]. However, more detailed mapping studies are needed to find closely linked markers and genes associated with sex determination. If homogametic females can be easily identified there is potential to use them as parents to yield completely sexually uniform heterogametic female offspring [38]. Although some markers associated with sex
determination have been identified [38], little is known about candidate genes, mechanism or map regions associated with the sex of crustaceans.

Here we undertake the first comprehensive genome scan for QTL associated with resistance to WSSV and for the sex-determining locus in *P. monodon*. A new WSSV challenge testing protocol that aims to deliver more accurate disease resistant phenotypes is devised and utilised. A set of 3959 linkage mapped transcribed gene SNPs are used to genotype 1038 sexed individuals derived from 7 full-sibling families challenged-tested for WSSV.

**Results**

**Challenge tests**

Shrimp survived on average $57.2 \pm 12.0$ SD hrs post challenge and a spread of *hours of survival* was observed within families (eg. ranging between 30 and 90 hours for the upper and lower 40 percentiles genotyped from families B, F and G Figure 1). No mortality was observed in the control group injected with saline buffer.

**Genetic parameters associated with white spot syndrome virus resistance**

Neither sex nor time of challenge (family) had significant effects on time to death in the model (Table 1, all 95% confidence intervals overlap zero).

**Linkage disequilibrium**

Mean and median values of LD, measured as $r^2$ between adjacent markers for the 3961 genome-wide distributed SNPs used in this study, were 0.35 and 0.30, respectively.

**QTL for WSSV resistance – GWAS and interval mapping analysis**

The quality control steps excluded all markers with non-Mendelian inheritance and all individuals unassigned with parentage analysis, leaving 3959 markers and 1038 individuals for analysis. For the FASTA and GRAMMAS GWAS analysis the additional quality control steps excluded 135 markers and 17 individuals with a call rate of less than 95%, 5 individuals with high autosomal heterozygosity (FDR <1%) and 4 individuals with identity by descent ≥0.95. No markers or individuals with a call rate less than 0.1 and minor allele frequency <0.24% were detected. After the quality control, 3824 markers and 1019 individuals were selected for the FASTA and GRAMMAS analysis.

Ten significant QTL for WSSV resistance (*hours of survival* post-WSSV infection, $P < 0.05$ after Bonferroni correction) were detected on linkage groups 2, 3, 5, 6, 17, 18, 19, 22, 27 and 43 (Table 2, Figures 2 and 3). Eight SNPs (51997_2402, 41442_21, 45605_1545, 29124_228, 44821_270, 50096_1789, 18472_352, 27976_64 on linkage groups 2, 3, 5, 6, 18, 19, 22, and 43 respectively) showed significant genome wide associations, and three regions (between SNPs 50756_3741 and 46539 on LG6, SNPs 25133_74 and 36717_243 on LG17 and SNPs 18687_338 and 3729_523 on LG27) showed significant linkage with *hours of survival*. These SNPs occurred in transcripts for genes encoding runt protein 1a, flagellar hook-length control protein, ubiquitin domain-containing protein ubfd1, paired-like homeodomain transcription factor 3, ankyrin repeat and many unannotated genes. Box plots of *hours of survival* post-WSSV infection for individuals with alternative genotypes for two informative SNPs in the vicinity of the QTL detected on linkage group 17, and for GWAS significant SNPs on linkage groups 18 and 22 (Figure 4), show patterns indicating additive gene effects for these QTL.

Some of the SNPs associated with QTL were found to map within or close to genes with putative immune functions of interest (Tables 2 and 3, Additional file 1). For example, the SNP marking a QTL at position 61.8 cM on linkage group 2 (51997_2402, $P < 0.05$ after Bonferroni correction for the QFAM test), occurred in a transcript that shared high homology to a gene encoding runt protein 1a in the signal crayfish *Pacifastacus leniusculus*. SNP 24034_664 at 47.3 cM on LG 2 in a transcript with homology to the proteasome (macropain) 26 s gene maps in the middle of a broad 41 cM region containing several SNPs in transcripts showing suggestive and significant associations with *hours of survival* after WSSV infection (including SNP 51997_2402, $P < 0.05$ after Bonferroni correction, at 61.8 cM).

Variation at SNP 45605_1545 in a transcript with homology to a gene encoding ubiquitin domain-containing protein ubfd1 on LG 5 was associated with *hours of survival* (FASTA $P < 0.05$ after Bonferroni correction). The SNP 40050_2030 occurs in a transcript with homology to a gene encoding 26 s proteasome subunit s9 and maps 4.4 cM from SNP 33044_1018 (suggestive association on LG6) and 0.6 cM from a predicted QTL (GridQTL, position 39 cM, $P < 0.05$ chromosome-wide significance). The SNP 49912_5110 which occurs in a transcript with homology to the mitogen activated protein kinase gene, mapped 2.3 cM from the QTL position detected by GridQTL analysis on LG17 ($P < 0.01$ genome-wide significance). The SNP 52376_14757 occurs in a transcript that shared high homology to a gene encoding ubiquitin gene and maps 3.9 cM from SNP 51997_2402 (suggestive association) and 0.6 cM from a predicted QTL (GridQTL, position 39 cM, $P < 0.05$ chromosome-wide significance). The SNP 49912_5110 which occurs in a transcript with homology to the mitogen activated protein kinase gene, mapped 2.3 cM from the QTL position detected by GridQTL analysis on LG17 ($P < 0.01$ genome-wide significance). The SNP 52376_14757 occurs in a transcript that is homologous to the hect e3 ubiquitin gene and maps 3.9 cM from SNP 51997_2402 (suggestive association) and 0.6 cM from a predicted QTL (GridQTL, position 39 cM, $P < 0.05$ chromosome-wide significance). The SNP 52376_14757 occurs in a transcript that shared high homology to a gene encoding ubiquitin conjugating enzyme 7 interacting protein, map 12.8 and 0.6 cM respectively from SNP 50096_1789 ($P < 0.05$ after Bonferroni correction, test QFAM) at 82.4 cM.
on LG19. Three genes encoding proteins with putative immune function map near to SNP 18472_352 ($P < 0.01$ after Bonferroni correction, QFAM test) at 27.9 cM on LG22, SNP 52279_11861 which also maps to 27.9 cM on LG 22 and which occurs in a transcript with homology to the serine-threonine protein kinase gene, SNP 42578_2554 which occurs in a transcript with homology to a gene encoding mitogen-activated protein kinase-binding protein 1 which is 1.9 cM distant and SNP 50961_705 which occurs in a transcript showing homology to a gene encoding IGF2 mRNA binding protein and is 2.4 cM distant.

Figure 1 Plot of hours of survival among progeny genotyped from 7 full-sibling families (A-G).
Association with sex on LG30
In all, 15 SNP markers were significantly associated with sex, (5 at \(P < 0.01\) and 10 at \(P < 0.001\) significance levels after Bonferroni correction, Additional file 2, Figure 5A and B). All significant associations mapped to a broad 43 cM interval of LG30 between positions 21.7 and 64.7 cM. The three markers with the strongest association mapped to an interval of 0.8 cM (positions 43.5 - 44.3 cM, SNPs 49245_2916, 49087_997 and 49482_526). Most significant was SNP 49245_2916 (\(P = 1.9E-49\)) which occurs in a gene encoding G7-c-like protein and von Williebrand factor A domain-containing protein 7 (Additional file 2).

The sex locus was predicted to map to 45 cM on LG30 by the GridQTL interval mapping analysis (\(P < 0.001\) genome-wide significance, Figure 5B).

The pattern of segregation of this locus to male and female offspring fits what would be expected for a locus associated with sex determination, assuming that female P. monodon are the heterogametic sex (Figure 5C and D). Eighty-seven percent of males (out of 483 genotyped) were homozygous AA for SNP 49245_2916 across the families (the allele frequency of A and G alleles was 0.93 and 0.07 respectively, n = 966) whereas ninety percent of females (541 genotyped) were heterozygous AG. Of the males that were not AA, 13% were AG, and less than 1% were GG genotypes. The GG males were only detected in one family (3/74 individuals in family 4 which also contained a high proportion, 30/74, of AG males). Most other families contained a low proportion of AG males, except two families (2 and 6) where all males were AA genotypes. Of the females that were not AG, 5% were AA and 5% were GG. The GG females were only detected in one particular full-sibling family (family 4 with 26/64 female genotypes recorded as GG). All families contained low numbers of AA females, except family 5 in which 56/56 females were AG. Also mapping to this region (at 44.3 cM) is SNP 43522_2279 which occurs in a transcript with homology to the feminisation-1 gene (fem-1 homolog c) in the nematode Caenorhabditis elegans (69% homology, contig length 3820 bases, Additional file 1 and Additional file 2).

Discussion
Invertebrates rely on innate immune systems to recognise and respond to foreign agents. Resistance to disease is a complex quantitative trait that is likely to be regulated by the additive effects of many genes, epigenetics and by the environment. In contrast, sex, which is measured as a binomial qualitative trait, is likely to be determined by the action of a few genes mapping to a specific area of one linkage group. Variation affecting disease resistance or sex could act by changing the regulation of gene expression or by leading to modifications of the protein product and consequent function. The SNPs developed for this study were detected among shrimp sourced from the east coast of India and Andaman Islands [39]. In developing SNPs we included RNA from three individuals that had survived a severe WSSV outbreak on a farm in Bapatla. These survivors were included in the present study to improve the chance of detecting SNP variants that are associated with resistance to WSSV. All the SNPs used in the study occur in transcribed genes (ie. cSNPs).

The challenge test experiment used in this study which lodged shrimp in individual baskets was designed so that all shrimp could be collected and sampled within 1 hour of death and to prevent secondary infection (transmitted with cannibalism). Although the time from infection with WSSV to death is rapid, a controlled route of infection and dosage was chosen to prolong the overall time frame of the experiment as much as possible and to give a spread of hours of survival. Large full-sibling families (146 offspring per family on average) and frequent observation

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Table 1 Summary of MCMCglmm analysis under an animal model of days survival after WSSV experimental challenge

| Parameter | Mean | Lower | Upper | Effective sample | pMCMCglmm |
|-----------|------|-------|-------|-----------------|-----------|
| (Intercept) | 57.4 | 44.7  | 68.0  | 1522            | <7e-04*** |
| sexM | 0.3 | -1.0 | 1.7 | 1400 | 0.679 |
| pedD5 | -1.3 | -13.9 | 18.4 | 1400 | 0.64 |
| pedE5 | -3.6 | -17.9 | 14.9 | 1400 | 0.59 |
| pedF5 | -5.7 | -20.8 | 11.6 | 1400 | 0.406 |
| pedG5 | -9.3 | -24.6 | 8.4 | 1400 | 0.196 |
| pedH5 | 9.5 | -9.0 | 24.7 | 1400 | 0.201 |
| pedH5a | 3.4 | -12.4 | 19.7 | 1585 | 0.6 |

Sex (male sexM) and family (pedD5, pedE5, pedF5, pedG5, pedH5 and pedH5a) fitted as fixed effects. Mean, mean of posterior distribution.

***P < 0.001.
Table 2: Suggestive and significant QTL for trait hours of survival after WSSV challenge detected using PLINK (QFAM total) and GenAbel (FASTA and GRAMMAS) analyses in 7 _P. monodon_ families

| LG | Pos | SNP   | Test     | N | Effect     | Stat  | P-value | Sig   | GeneID                          |
|----|-----|-------|----------|---|------------|-------|---------|-------|---------------------------------|
| 1  | 60.3| 39454_862 | GRAMMAS  | 1007 | 1.14(0.53) | 4.66  | 0.0032  | unknown                      |
| 2  | 0   | 47460_2015 | QFAM    | 1024 | −4.538     | 56.81 | 0.0094  | dna p58 subunit               |
| 2  | 21.6| 52022_4578 | QFAM    | 1020 | 6.351      | 83.1  | 0.0049  | plasminogen activator inhibitor 1 |
| 2  | 24.5| 50149_330  | FASTA   | 983  | 3.32(1.03) | 10.49 | 0.0014  | thyroid transcription factor 1-associated protein 26-like protein |
| 2  | 24.5| 50149_330  | GRAMMAS | 983  | 1.65(0.71) | 5.44  | 0.0015  | thyroid transcription factor 1-associated protein 26-like protein |
| 2  | 30.5| 52064_948  | GRAMMAS | 1007 | 0.95(0.49) | 3.79  | 0.008   | polymerase I polypeptide 194kda |
| 2  | 36.6| 36607_579  | GRAMMAS | 1006 | −0.91(0.46) | 3.94  | 0.0068  | unknown                        |
| 2  | 53.5| 28698_101  | GRAMMAS | 965  | −1.01(0.48) | 4.4   | 0.0042  | unknown                        |
| 2  | 53.5| 28698_101  | FASTA   | 965  | −1.75(0.65) | 7.22  | 0.0082  | unknown                        |
| 2  | 61.8| 51997_2402 | QFAM    | 1023 | −5.49      | 139.2 | 0.0009  | runt protein 1a                |
| 2  | 62.5| 35650_1855 | FASTA   | 1004 | 2.11(0.79) | 7.23  | 0.0081  | unknown                        |
| 2  | 62.5| 35650_1855 | QFAM    | 1018 | 5.408      | 100.5 | 0.0085  | unknown                        |
| 2  | 14.6| 38676_1386 | QFAM    | 1021 | −4.2       | 41.79 | 0.0069  | actin-binding rho-activating   |
| 3  | 29.5| 41442_2163 | GRAMMAS | 1006 | −1.35(0.55) | 5.99  | 0.0008  | flagellar hook-length control protein flik |
| 3  | 29.5| 41442_2163 | FASTA   | 1006 | −1.88(0.68) | 7.62  | 0.0066  | flagellar hook-length control protein flik |
| 5  | 21.2| 45605_1545 | FASTA   | 1007 | 7.34(1.94) | 14.26 | 0.0002  | ubiquitin domain-containing protein ubfd1 |
| 5  | 21.2| 45605_1545 | GRAMMAS | 1007 | 2.42(1.06) | 5.22  | 0.0018  | ubiquitin domain-containing protein ubfd1 |
| 5  | 21.9| 35133_160  | FASTA   | 991  | −2.98(0.96) | 9.56  | 0.0023  | unknown                        |
| 5  | 21.9| 35133_160  | GRAMMAS | 991  | −1.42(0.65) | 4.81  | 0.0028  | unknown                        |
| 5  | 22.3| 44076_3116 | FASTA   | 1004 | −1.15(0.49) | 5.4   | 0.0015  | vacuolar proton atpase          |
| 5  | 22.3| 44076_3116 | GRAMMAS | 1004 | −1.82(0.65) | 7.79  | 0.006   | vacuolar proton atpase          |
| 5  | 87  | 45237_316  | GridQTL  | 1024 | 11.96(2.44) | 24   | 0.0048  | unknown                        |
| 5  | 96.5| 30527_111  | QFAM    | 992  | −1.88(0.68) | 7.67  | 0.0064  | unknown                        |
| 6  | 17.3| 29124_228  | FASTA   | 1007 | 4.82(1.34) | 12.88 | 0.0004  | paired-like homeodomain transciption factor 3 |
| 6  | 17.3| 29124_228  | GRAMMAS | 1007 | 1.49(0.71) | 4.42  | 0.0042  | paired-like homeodomain transciption factor 3 |
| 6  | 39  | 50756_3741-46539_1081 | GridQTL | 1024 | 11.25(2.32) | 23.54 | 0.0098  | erythrocyte band 7 integral membrane protein |
| 6  | 42.8| 33044_1018 | FASTA   | 1007 | 3.65(1.15) | 10.07 | 0.0018  | abb73282reverse transcriptase   |
| 8  | 45.4| 52776_1335 | QFAM    | 1021 | 5.849      | 56.79 | 0.0036  | multidrug resistance-associated protein 14 |
| 9  | 10.4| 42679_345  | QFAM    | 1007 | 4.823      | 81.8  | 0.0039  | 26 s protease regulatory subunit |
| 9  | 59.9| 48064_77   | QFAM    | 1024 | 4.807      | 126.6 | 0.0072  | actin-binding homolog 1         |
| 11 | 24.7| 60951_72   | FASTA   | 1007 | −3.46(1.14) | 9.2   | 0.0028  | usick-kaufman syndrome          |
| 11 | 24.7| 60951_72   | GRAMMAS | 1007 | −1.37(0.68) | 4.05  | 0.0061  | unknown                        |
| 11 | 38.1| 46551_1072 | QFAM    | 1024 | 5.642      | 135.6 | 0.0016  | multidrug resistance-associated protein 14 |
| 11 | 59.4| 23272_344  | FASTA   | 1007 | 3.52(1.33) | 7.01  | 0.0091  | usick-kaufman syndrome          |
| 13 | 18  | 29098_2532 | QFAM    | 1024 | 3.95       | 50.35 | 0.0076  | actin-binding homolog 1         |
| 14 | 49.5| 40042_2041 | QFAM    | 1021 | 4.192      | 46.08 | 0.0046  | unknown                        |
| 15 | 27.2| 32667_1134 | QFAM    | 1023 | −5.196     | 104.9 | 0.0067  | usick-kaufman syndrome          |
| 15 | 47.8| 44399_644  | FASTA   | 1007 | 2.28(0.78) | 8.61  | 0.0039  | unknown                        |
Table 2: Suggestive and significant QTL for trait hours of survival after WSSV challenge detected using PLINK (QFAM total) and GenAbel (FASTA and GRAMMAS) analyses in 7 *P. monodon* families (Continued)

| Chromosome | SNP Position | Analysis Method | Tag ID | Haploscore | $p$-value | Description |
|------------|--------------|----------------|--------|------------|------------|-------------|
| 16 11 | 42291_720 | GRAMMAS | 1007 | 2.13(0.93) | 5.24 | 0.0018 | adp-ribosylation factor-like 2 binding protein |
| 16 11 | 42291_720 | FASTA | 1007 | 4(1.34) | 8.91 | 0.0033 | adp-ribosylation factor-like 2 binding protein |
| 16 11 | 38195_1528 | FASTA | 1003 | 2.46(0.84) | 8.52 | 0.0041 | fanconi anemia group a protein homolog |
| 16 23.4 | 45647_100 | QFAM | 1018 | −4.662 | 56.63 | 0.009 | glutamyl-tRNA amidotransferase subunit |
| 16 38.1 | 35920_135 | QFAM | 1012 | 3.142 | 8.91 | 0.0033 | adp-ribosylation factor-like 2 binding protein |
| 16 39.2 | 5999_123 | FASTA | 1007 | 2.75(1.01) | 7.34 | 0.0076 | unknown |
| 17 8.3 | 39727_708 | GRAMMAS | 1007 | 0.82(0.41) | 4.01 | 0.0063 | unknown |
| 17 26.7 | 26178_2213 | QFAM | 1018 | 4.67 | 78.32 | 0.0067 | bobby sox |
| 17 29 | 47941_2759 | FASTA | 1007 | 2.75(1.01) | 7.34 | 0.0076 | alsin isoform 2 |
| 17 54 | 25133_74 to 36717_243 | GridQTL | 1024 | 22.90 | 89.81 | 0.0001 ** | unknown |
| 18 15.1 | 44821_270 | FASTA | 1006 | 7.26(1.85) | 15.34 | 0.0001 ** | unknown |
| 18 15.1 | 44821_270 | GRAMMAS | 1006 | 3.35(1.2) | 7.83 | 0.0001 ** | unknown |
| 18 81.5 | 24411_90 | GRAMMAS | 1006 | −0.9(0.46) | 3.83 | 0.0076 | unknown |
| 19 34.8 | 35006_276 | QFAM | 1024 | 5.931 | 92.83 | 0.0011 | alanyl-tRNA synthetase |
| 19 44.5 | 14555_138 | QFAM | 1021 | 6.616 | 125.5 | 0.006 | unknown |
| 19 70.9 | 51029_2543 | QFAM | 1023 | −3.578 | 40.82 | 0.0029 | unknown |
| 19 82.4 | 50996_1789 | QFAM | 1021 | 5.243 | 77.12 | 0.0005 * | insulin receptor substrate 1 |
| 20 23.1 | 36484_493 | QFAM | 1007 | 3.48(1.31) | 7.01 | 0.0091 | unknown |
| 20 63.1 | 42447_399 | GRAMMAS | 1007 | −1.14(0.57) | 4.04 | 0.0062 | mitochondrial ribosomal protein l2 |
| 20 63.1 | 42447_399 | FASTA | 1007 | −2.26(0.84) | 7.26 | 0.008 | unknown |
| 21 20.1 | 47262_891 | GRAMMAS | 1006 | −0.9(0.45) | 4.19 | 0.0053 | myostatin 1b |
| 21 20.1 | 47262_891 | FASTA | 1021 | 6.616 | 125.5 | 0.006 | myostatin 1b |
| 21 20.1 | 47262_891 | QFAM | 1024 | −1.75(0.66) | 7.17 | 0.0084 | myostatin 1b |
| 21 26 | 30265_1829 | FASTA | 1007 | 1.96(0.68) | 8.44 | 0.0042 | unknown |
| 21 26 | 30265_1829 | GRAMMAS | 1007 | 0.88(0.43) | 4.2 | 0.0052 | unknown |
| 21 28.5 | 29404_373 | GRAMMAS | 1007 | 1.26(0.63) | 4.05 | 0.0061 | unknown |
| 21 28.8 | 19638_158 | QFAM | 1011 | 4.032 | 45.59 | 0.008 | unknown |
| 21 89.5 | 40988_772 | GRAMMAS | 1007 | −0.98(0.35) | 3.77 | 0.0082 | unknown |
| 21 9.1 | 52299_3858 | GRAMMAS | 1003 | −1.08(0.55) | 3.8 | 0.0079 | c12orf66-like |
| 22 20.8 | 25410_46 | GRAMMAS | 986 | −1.13(0.51) | 4.96 | 0.0024 | unknown |
| 22 27.9 | 18472_352 | FASTA | 1007 | −2.11(0.76) | 7.69 | 0.0063 | unknown |
| 22 27.9 | 18472_352 | GRAMMAS | 1007 | −0.98(0.49) | 3.97 | 0.0066 | unknown |
| 22 27.9 | 18472_352 | QFAM | 1024 | −5.815 | 104.9 | 0.0001 ** | unknown |
| 23 83.5 | 41044_732 | QFAM | 1024 | 5.885 | 110.7 | 0.0043 | unknown |
| 24 0.4 | 49156_279 | GRAMMAS | 1007 | 1.11(0.55) | 4.13 | 0.0056 | hsp90 |
| 24 0.4 | 49156_279 | FASTA | 1007 | 2.21(0.81) | 7.43 | 0.0073 | hsp90 |
| 24 50.3 | 51251_2007 | QFAM | 1018 | −5.541 | 141.1 | 0.0023 | cub-serine protease |
| 25 0 | 44977_264 | QFAM | 1024 | 4.927 | 61.93 | 0.004 | unknown |
| 26 0.6 | 52048_2568 | GRAMMAS | 992 | −1.12(0.57) | 3.8 | 0.0079 | adenosine monophosphate-protein transferase ficd homolog |
| 26 0.6 | 52048_2568 | QFAM | 1009 | −6.31 | 89.68 | 0.002 | adenosine monophosphate-protein transferase ficd homolog |
Table 2 Suggestive and significant QTL for trait hours of survival after WSSV challenge detected using PLINK (QFAM total) and GenAbel (FASTA and GRAMMAS) analyses in 7 P. monodon families (Continued)

| Family | Chromosome | Position | Gene Symbol | Gene Name | Total LOD | p-Value | Annotation
|--------|------------|----------|-------------|-----------|-----------|---------|------------------------|
| 26     | 8.5        | 44451_587| QFAM        | 1023      | 5.597     | 0.0021  | unknown                |
| 26     | 58.9       | 33059_367| QFAM        | 1024      | −5.422    | 0.0059  | unknown                |
| 26     | 18687_338-33729_523 | GridQTL | 1024 | 8.64(2.39) | 13.04 | 0.018 * | unknown                |
| 27     | 52.7       | 47625_1438| GRAMMAS    | 1006      | −0.99(0.52) | 3.7   | 0.0087 | unknown                |
| 27     | 63.6       | 33004_1869| GRAMMAS    | 1007      | −1.26(0.59) | 4.51 | 0.0038 | unknown                |
| 27     | 91.9       | 43302_1775| FASTA      | 1007      | 2.2(0.8)  | 7.54   | 0.0069 | unknown                |
| 27     | 91.9       | 43302_1775| GRAMMAS    | 1007      | 0.98(0.51) | 3.73  | 0.0085 | dead box atp-dependent rna helicase |
| 28     | 20.8       | 51400_2931| GRAMMAS    | 1006      | 1.32(0.63) | 4.38  | 0.0043 | unknown                |
| 28     | 20.8       | 51400_2931| QFAM       | 1019      | 4.917     | 43.37  | 0.0089 | unknown                |
| 28     | 30.6       | 47112_509 | FASTA      | 1006      | −2.82(0.87) | 10.63 | 0.0013 | chorion peroxidase      |
| 29     | 29.7       | 52042_128 | QFAM       | 1022      | 4.654     | 94.7   | 0.0065 | multiple c2 domain and transmembrane region |
| 29     | 44         | 43412_2186| GRAMMAS    | 1007      | −1.35(0.71) | 3.58  | 0.0099 | gpi-anchor transamidase |
| 30     | 77.3       | 52990_1729| QFAM       | 1016      | 4.115     | 60.01  | 0.0047 | breast carcinoma-amplified sequence 3 homolog isoform 1 |
| 31     | 14.7       | 36096_367 | FASTA      | 1007      | 3.82(1.24) | 9.49  | 0.0024 | nucleostemin-like protein |
| 32     | 36.6       | 47777_1061| FASTA      | 1002      | −1.82(0.7) | 6.85  | 0.01   | exonuclease 3–5 domain-containing protein 2 isoform 1 |
| 32     | 36.6       | 47777_1061| QFAM       | 1012      | −4.716    | 76.83  | 0.0035 | exonuclease 3–5 domain-containing protein 2 isoform 1 |
| 34     | 32.3       | 24101_537 | GRAMMAS    | 1007      | −1.56(0.72) | 4.67  | 0.0032 | zinc finger protein 64-like |
| 34     | 32.3       | 24101_537 | FASTA      | 1007      | −2.68(0.99) | 7.25  | 0.008  | zinc finger protein 64-like |
| 36     | 29.6       | 30057_491 | QFAM       | 1023      | −5.252    | 75.55  | 0.009  | unknown                |
| 36     | 32.1       | 49829_3826| QFAM       | 962       | −4.221    | 71.99  | 0.0044 | unknown                |
| 36     | 57.6       | 50839_3313| GRAMMAS    | 1007      | 1.72(0.81) | 4.54  | 0.0037 | transcriptional enhancer factor tef- |
| 36     | 57.6       | 50839_3313| FASTA      | 1007      | 3.08(1.14) | 7.33  | 0.0077 | transcriptional enhancer factor tef- |
| 38     | 36.1       | 35013_386 | FASTA      | 1007      | −2.31(0.72) | 10.32 | 0.0016 | unknown                |
| 38     | 36.1       | 35013_386 | GRAMMAS    | 1007      | −0.88(0.42) | 4.36  | 0.0044 | unknown                |
| 38     | 66.9       | 17589_451 | GRAMMAS    | 1004      | 1.67(0.82) | 4.12  | 0.0056 | unknown                |
| 39     | 0.2        | 35101_271 | QFAM       | 1021      | 4.295     | 57.77  | 0.0045 | unknown                |
| 39     | 51.2       | 49386_1117| QFAM       | 1024      | −5.859    | 109.8  | 0.0086 | phospholipase c gamma |
| 39     | 59.4       | 36972_442 | FASTA      | 1004      | −1.85(0.66) | 7.82  | 0.0059 | unknown                |
| 39     | 59.4       | 36972_442 | GRAMMAS    | 1004      | −0.83(0.42) | 3.86  | 0.0074 | unknown                |
| 40     | 22.9       | 51885_4402| QFAM       | 1024      | 5.687     | 50.84  | 0.007  | chromodomain-helicase-dna-binding protein 1 |
| 40     | 68.1       | 11637_107 | QFAM       | 1020      | −7.046    | 129.7  | 0.0083 | non-lysosomal glucosylceramidase |
| 41     | 1.6        | 26900_757 | QFAM       | 1021      | −7.651    | 113.7  | 0.0079 | unknown                |
| 42     | 59         | 35645_15  | GRAMMAS    | 1005      | 0.99(0.46) | 4.73  | 0.003  | unknown                |
| 43     | 0.4        | 27976_64  | GRAMMAS    | 990       | −1.4(0.52) | 7.3   | 0.0002 | unknown                |
| 43     | 0.4        | 27976_64  | FASTA      | 990       | −1.84(0.64) | 8.39  | 0.0044 | unknown                |
| 44     | 0          | 38601_555 | FASTA      | 1007      | −2.39(0.88) | 7.41  | 0.0074 | unknown                |
| 44     | 0          | 38601_555 | GRAMMAS    | 1007      | −1.1(0.57) | 3.77  | 0.0081 | unknown                |
were also employed to give a strong power for detecting QTL.

Both the linkage and GWAS analyses detected significant QTL associated with hours of survival after WSSV infection. For three of the four QTL detected by linkage analysis, closely mapping SNPs with suggestive associations were detected by GWAS analysis (on linkage groups 5, 6 and 27, Table 2). Fewer QTL were detected using linkage analysis than using GWAS. While linkage analysis relies on the segregation of alleles within families, GWAS correlates the occurrence of SNP alleles with phenotypes across the population. Comparison of linkage analysis and GWAS has shown that GWAS, where all SNPs are fitted simultaneously as random effects, has greater power to discriminate linked QTL [40], especially those of limited or modest sized effects [41]. The sensitivity of linkage analysis is affected by the number of parents that are segregating for the QTL and neighbouring SNP loci and by the extent of linkage among SNPs mapping in the vicinity of the QTL. The sensitivity of GWAS depends on the existence of linkage disequilibrium between the QTL and single SNP loci (which, to some extent, is dependent on the number of SNPs tested) and on the existence of SNPs sharing a similar allele conformation to that of the QTL. It has been found by other studies that the two types of analyses generally yield inconsistent results, but can agree if the differences between the two methods (caused by differences in the precision for mapping QTL location, ability to account for multiple linked QTL and due to over estimation of what are sometimes modelled as fixed SNP effects), are accounted for [40].

For the GWAS analyses, the GRAMMAS and FASTA results were often in agreement, while the results of QFAM analysis were less often in agreement with GRAMMAS or FASTA. For instance, SNP 18472_352 on LG22 was found to be associated with hours survival by the QFAM test ($P < 0.01$ after Bonferroni correction), but was found to be suggestively associated with the trait by the GRAMMAS and FASTA tests. Similarly, SNP 51997_2402 on LG2 was associated with hours survival for the QFAM test ($P < 0.05$ after Bonferroni) and a closely mapping SNP was suggestively associated using the FASTA test. No agreement for the significant association detected by QFAM at position 82.4 cM was found by GRAMMAS or FASTA tests across LG19. Whereas, significant associations detected on linkage groups 3, 5, 18 and 43 by GRAMMAS or FASTA were supported by corresponding suggestive or significant associations by FASTA or GRAMMAS respectively for the same SNP. FASTA and GRAMMAS, which use genomic control to infer genetic relations from genomic data, and thereby account for the true genealogy (population structure and all levels of relationships), are thought to be superior to methods such as QFAM, which makes use of the observed genealogy (observed parent-offspring relationships in our study) [42].

### Candidate genes mapping to QTL regions

Several of the SNPs directly associated, or closely linked to WSSV resistance QTL, were found to occur in transcripts that share homology to genes with putative immune functions. Some of the genes, such as heat shock protein 21, c-type lectin and serine-threonine specific protein kinase, have been implicated in affecting the WSSV resistance of crustaceans in other studies [18,20,43-45]. Some are components of gene pathways, such as the ubiquitination pathway, which have been found to affect the pathogenesis of WSSV [46,47].

### The ubiquitin proteasome pathway

The ubiquitin proteasome pathway has been shown to play an important role in immune defence and more specifically proteasome I is presumed to be involved in intracellular antibody-mediated proteolysis of antibody-bound viruses [48]. Six SNPs in transcripts with homology to proteasome encoding genes of interest were either directly or closely mapped to QTL for WSSV resistance (Table 3), including SNP 24034_664 in a transcript with homology to the proteasome (macropain) gene which was 14.1 cm from SNP 51997_2402 ($P < 0.05$ after Bonferroni correction, LG2), SNP 23272_344 in a transcript with homology to the 26 s protease regulatory subunit gene (suggestive association), SNP 40050_2030 in a transcript with

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**Table 2** Suggestive and significant QTL for trait hours of survival after WSSV challenge detected using PLINK (QFAM total) and GenAbel (FASTA and GRAMMAS) analyses in 7 P. monodon families (Continued)

| 44 | 3.2 | 42369_480 | QFAM | 1024 | −5.011 | 91.99 | 0.0058 | tbc1 domain family member 14 isoform a |
|---|---|---|---|---|---|---|---|---|
| 44 | 26 | 51212_1738 | QFAM | 1024 | 6.066 | 107 | 0.0063 | sodium bicarbonate transporter-like protein 11 |
| 44 | 40.4 | 20208_30 | GRAMMAS | 1007 | 1.55(0.79) | 3.8 | 0.0078 | unknown |

LG, linkage group; Pos, location on LG in centimorgans; N, number of progeny and parents analysed; Effect, allele substitution effect of the minor allele with standard error in parenthesis (FASTA, GRAMMAS and GridQTL); Beta (QFAM); Stat, test statistic linear regression coefficient for QFAM, chi-square with one degree of freedom for FASTA and GRAMMAS analyses, F-statistic for GridQTL; P, point-wise empirical $P$-value (QFAM), permuted $P$-value with one degree of freedom corrected for inflation factor lambda (FASTA and GRAMMAS) or chromosome-wide $P$ search with permutation and bootstrap analysis (GridQTL); Sig, significance after Bonferroni correction (*$P < 0.05$; **$P < 0.01$). GenID, closest SNP homology from BLAST. Tests were considered suggestive when $P < 0.01$ before Bonferroni correction.
homology to the 26 s proteasome subunit s9 gene which maps 0.6 cM from a QTL position predicted by linkage analysis ($P < 0.05$ chromosome-wide significance on LG6), SNP 17687_140 in a transcript with homology to the proteasome subunit alpha type-7 gene which was 0.5 cM from SNP 32667_1134 (suggestive association with *hours of survival* on LG15), SNP 48349_91 in a transcript with homology to the proteasome (macropain) 26 s non-2 gene which maps 12.7 cM from SNP 50096_1789 ($P < 0.05$ after Bonferroni correction, LG19) and SNP 49666_3836 in a
transcript with homology to the 26 s proteasome non-
ATPase regulatory subunit 11-like gene which maps to the
same position as SNP 43412_2186 (suggestive association
at 44 cM on LG29).

Modulation of the host ubiquitin proteasome pathways
by viral proteins is thought to affect viral pathogenesis, and
four proteins have been identified in the WSSV (WSSV199,
WSSV222, WSSV249 and WSSV403) [49-52] which inter-
act with the *P. monodon* ubiquitination pathway (e.g. with
conjugating enzyme (E2) in shrimp) and act as viral E3
ubiquitin protein ligases to inhibit apoptosis and affect viral
pathogenesis [46,47]. Injection of recombinant *Fennerope-
naeus chinensis* ubiquitin-conjugating enzyme E2 has been
shown to reduce the mortality of shrimp challenged with
WSSV, inhibit replication of WSSV and bind to (and ubi-
quitinate) WSSV RING domain-containing proteins [53],
and ubiquitin C expression is up-regulated when *F. chinensis*
are challenged by WSSV [54]. It follows that variation in
the structure or expression of E3 ubiquitin-protein ligase,
ubiquitin conjugating enzyme (E2) or other enzymes in-
volved in the ubiquitin proteasome pathway, could be
important in affecting the resistance or susceptibility of
*P. monodon* to WSSV. Variation in a SNP in a transcript with
homology to the ubiquitin domain-containing protein
ubfd1 gene (45605_1545) mapping to 21.2 cM along
linkage group 5 was found to be associated with WSSV
resistance in this study (P < 0.05 after Bonferroni
for the FASTA test). The SNPs in nine other transcripts
with homology to genes involved in the ubiquitin prote-
asome pathway (two forms of e3 ubiquitin-protein ligase,
two forms of hect e3 ubiquitin, ubiquitin carboxyl-terminal
hydrolase 47, ubiquitin-conjugating enzyme e2 c, ubiquitin-
conjugating factor e4, ubiquitin-conjugating enzyme 7
interacting protein and ubiquitin protein ligase, Table 3)
were all found to show suggestive associations or to map
closely to other SNPs significantly or suggestively associated
with hours of survival after WSSV challenge in this study.

Lymphocyte function and heat shock proteins

Interleukin enhancer-binding factor 2 is a transcription
factor required for expression of the interleukin 2 gene
which regulates the activity of lymphocytes responsible
for immunity [55]. A SNP in a transcript with homology
to the gene coding for this factor was found to map
3 cM from a SNP (3927_708) with suggestive association
to hours of survival on LG17 (Table 3).

Heat shock proteins act as intercellular signalling
molecules for the regulation of the immune response
of many organisms, particularly with regard to lymphocyte
mediated responses [56]. The Hsp70-Hsp90 organizing

![Figure 3 GridQTL interval mapping F-test statistic plots for trait hours of survival across all linkage groups (A) and across LG17 (B).](image)
protein (Hop, SNP 45405_1355 at 26.7 on LG17, Table 3) is a co-chaperone that reversibly links HSP70 and HSP90, moderating chaperone activity. The expression of HSP70 and HSP90 increases in hemocyte and lymphoid organs when crustaceans (*Marsupenaeus japonicus* and *Procambarus clarkii*) are challenged with WSSV [43,44]. HSP21 is normally highly expressed in *P. monodon* tissues, but is down-regulated following infection with WSSV [20].

The TOLL pathway

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB, SNP 51361_1388 at 4.7 cM on LG 25, Table 3) is a rapid acting primary transcription factor which regulates the innate and adaptive immune cellular response to viral and other forms of infection. When pattern recognition toll-like receptors in T- or B-cells are activated, NF-kB enters the nucleus and up-regulates genes involved in development, maturation and proliferation (eg. type I interferon response genes). Large precursor molecules of NF-kB (p105 and p100) are processed by the ubiquitin/proteasome pathway which involves the degradation of ankyrin repeat c-terminal regions.

“Inappropriate” activation of NFkB has been linked to AIDS, whereas inhibition has been linked to disorders in immune cell development. The stimulation of activator protein 1 activity by mitogen-activated protein kinases is thought to elicit stress responses and promote cell survival and death in response to viral infection [57].

Mitogen activated protein kinases

Protein kinase signal transduction pathways, including mitogen-activated protein kinases, have been shown to have important roles in the regulation of cytokine gene expression [58-60], particularly interleukin-1, which is a potent inflammatory cytokine regulating host defence and immune responses [61]. Mitogen activated protein
kinases (MAP kinases) are involved in directing cellular responses to a range of stimuli including viral infection. Extracellular signal-regulated kinase is a type of serine-threonine specific protein kinase that is activated by WSSV in the early stage of infection, and when silenced or inhibited, reduces WSSV proliferation, and delays viral early gene transcription, in *L. vannamei* [45]. The SNPs in transcripts with homology to mitogen activated protein kinase, mitogen-activated protein kinase organisering factor 1, map kinase-activated protein kinase organiser 1, and ubiquitin protein ligase 3 are associated with putative immune function. Table 3 shows SNPs with homology to genes of putative immune function mapping near to QTL regions.

| LG | cM | SNP | GeneID | Length | Hits | E-value | Similarity |
|----|----|-----|--------|--------|------|---------|------------|
| 2  | 0, 21.6, 24.5, 30.5, 36.6, 53.5, 61.8* and 62.5 | 47.3 | 24034_664 proteasome (macropain) 26 s | 991 | 20 | 3.48E-45 | 62.15% |
| 5  | 21.2*, 21.9, 22.3, 87* and 96.5 | 21.2 | 45605_1545 ubiquitin domain-containing protein ubfd1 | 1764 | 20 | 5.32E-81 | 66.7% |
| 6  | 17.3* 39* and 42.8 | 38.4 | 40050_2030 26 s proteasome subunit s9 | 2299 | 20 | 2.33E-143 | 76.50% |
| 9  | 10.4 and 59.9 | 59.7 | 42539_708 E3 ubiquitin-protein ligase RAD18 | 1522 | 20 | 1.62E-51 | 46.90% |
| 11 | 24.7, 38.1 and 59.4 | 20.8 | 44253_2858 ubiquitin protein ligase | 3938 | 20 | 0 | 64.25% |

*P <0.05; **P <0.01 after Bonferroni correction. GeneID, identity allocated by blast2go using consensus annotations for the top hits. Length, length of query contig sequence. Hits, number of sequences found to match query (maximum 20). E-value, minimum e-value (probability of alignment occurring by chance) recorded for a hit. Similarity, percent mean similarity recorded across hits.
isoform, serine-threonine protein kinase, interleukin enhancer binding factor and mitogen-activated protein kinase-binding protein 1 were found to map near to SNPs showing suggestive and significant (LG17 GridQTL \( P < 0.01 \) genome-wide significance) associations with days survival on linkage groups 11, 16, 17, 22 and 28 (Table 3 and Figures 2 and 3).

The mRNA binding proteins, such as IGF2 mRNA binding protein (gene mapping 2.4 cM from SNP 18472_352, \( P < 0.01 \) after Bonferroni for the QFAM test, Table 3), play an important role in stabilizing mRNAs during cellular stress [62].

**Lectin**

Lectins are non-self-recognition factors thought to be involved in immune recognition and microorganism phagocytosis through opsonisation in crustaceans [63]. A SNP in a transcript with homology to C-type lectin (45153_220) maps 1.8 cM from SNP 27976_64 on LG43 (\( P < 0.05 \) after Bonferroni correction for the GRAMMAS test, Table 3). Tiger shrimp surviving more than 84 hrs post WSSV infection have been observed to have higher haemocyte expression of c-type lectin [18]. WSSV infected \( L. \) *vannamei* that are pre-challenged with WSSV shower higher haemocyte expression of c-type lectin than previously naïve individuals [17]. Lectin is also more highly expressed in the hepatopancreas of resistant \( L. \) *vannamei* [64], and in the haemocytes and hepatopancreas of resistant \( M. \) *japonicus* [65,66], than more susceptible individuals. C-type lectin-like domains have been detected in other genes such as PmAV, which are believed to be involved in conferring viral resistance in \( P. \) *monodon* [33].

**Runt protein**

The runt protein is up-regulated prior to haemocyte release and is known to be involved in haematopoiesis [67]. The RUNT-related transcription factors (eg. RUNX3/p33) play important roles in the development and differentiation

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**Figure 5** GridQTL interval mapping F-statistic plots over all linkage groups (A) and on LG30 (B) for the trait sex, and genotype frequency differences between male (C) and female (D) \( P. \) *monodon* for SNP 49245_2916 located at 43.5 cM on LG 30 which was found to be significantly associated with sex (\( P < 0.001 \) after Bonferroni correction).
of the immune system [68] and mutations in this gene are known to be associated with greater susceptibility to autoimmune disorders [69]. The expression of RUNT domain protein is 40% lower in Norwegian lobsters (Nephrops norvegicus) that are immunologically suppressed by high levels of manganese [70]. A SNP associated with WSSV resistance on LG2 (51997_2402, \( P < 0.05 \) after Bonferroni correction for the QFAM test, Table 2), occurred in a transcript which shared high homology to runt protein 1a in the signal crayfish Pacifastacus leniusculus.

Detection of markers associated with sex

Although sex determination is a simply inherited binary trait in most organisms, the precise genetic processes affecting sex determination have been found to be complex and diverse. SNP 43522_2279 occurs in a transcript for a protein known to be associated with greater susceptibility to autoimmune disorders [68] and mutations in this gene are known to be associated with greater susceptibility to autoimmune disorders [69]. The expression of RUNT domain protein is 40% lower in Norwegian lobsters (Nephrops norvegicus) that are immunologically suppressed by high levels of manganese [70]. A SNP associated with WSSV resistance on LG2 (51997_2402, \( P < 0.05 \) after Bonferroni correction for the QFAM test, Table 2), occurred in a transcript which shared high homology to runt protein 1a in the signal crayfish Pacifastacus leniusculus.

In summary, indications are that the markers identified by this study, could be useful for the purpose of identifying homogametic females. Detailed studies of mutations and phenotypes in candidate genes mapping in this region of linkage group 30, could lead us to a better understanding of the genetic mechanisms affecting sexual dimorphism in P. monodon. In other invertebrates such as C. elegans there are a diversity of molecules and control networks involved in sex determination [71]. The models for sex determination developed for C. elegans and other invertebrates such as Drosophila melanogaster will be informative.

Application to marker assisted or genomic selection

Further research is needed to predict the most effective means of using the markers identified here to assist the genetic improvement of WSSV resistance. Consideration needs to be given to the overall goals of the breeding programs to which marker information is applied. In 2001, Meuwissen et al. [76] devised a method for the prediction of total genetic value using genome-wide dense marker maps, without phenotypic information, which has otherwise become known as genomic selection (GS). With the development of new low-cost fully-automated genotyping technologies, use of genome wide dense marker maps, without phenotypic information, which has otherwise become known as genomic selection (GS). With the development of new low-cost fully-automated genotyping technologies, use of genome wide dense marker maps, without phenotypic information, which has otherwise become known as genomic selection (GS). With the development of new low-cost fully-automated genotyping technologies, use of genome wide dense marker maps, without phenotypic information, which has otherwise become known as genomic selection (GS).
in this and closely related species such as improving resistance to WSSV should be further evaluated. Hawaii) and the efficacy of marker assisted selection for breeding programs for applied to supplement genetic evaluations made by selective combination in one tonne fibre re-inforced plastic (FRP) tanks. The shrimp were fed on a diet consisting of squid and polychaete worms which facilitates maturation. From maturation trials, seven full-sib families were produced. The shrimp from these families were cultured in separate hapas in a pond to an injectable size of about 3 to 5 g in order to retain family identity. At this stage, approximately 20 juveniles were randomly collected from the hapas and transferred to the challenge test facility where they were introduced into a 4 t concrete cement tank. The shrimp were allowed to de-stress for a couple of days to overcome the transportation stress. From each lot of 200 shrimp, a sample of ten shrimp were collected at random and tested using the WSSV detection kit.

**WSSV challenge experiment**

A custom-made experimental facility, for preventing cannibalism, was fabricated for challenge studies to achieve recovery of all challenged shrimp. This facility consisted of multiple plastic baskets that were anchored to a support and lodged side-by-side at the same depth (just below the water surface) in a cement tank. Only one shrimp was housed in each basket during the experiment. Each basket had a lid for ease of placing or removing shrimp. The base of each basket had plastic wire mesh stitched to the sides such that feed pellets could be retained and faecal matter could easily pass through.

The muscle tissue from juvenile shrimp that were fed with WSSV-infected shrimp meat were used for extraction of WSSV virus following the protocol of [82]. The virus stock concentration was established as 1.04 X 10^7 copies per μl in a real-time standard curve experiment. Trials were undertaken to compare intramuscular and oral routes of challenge and it was observed that intramuscular injection gave consistent results compared to the venocatch method. Consequent to this finding, all the experimental shrimp were challenged with the WSSV virus following the intramuscular method. The shrimp were injected intramuscularly with 100 μl of 10^-5 dilution of virus stock using 1 mL tuberculin syringe. The virus was injected into the muscle tissue between the third and fourth abdominal segments on the lateral side. Extra care was exercised to avoid physical injury to the intestine and aorta running along the dorsal side and nerve cord running along the ventral side of the abdomen. After injection, the shrimp were retained in a 4 tonne cement tank for 6 hours to de-stress and to observe any mortality due to physical injury. De-stressed shrimp were then placed in individual baskets and monitored at hourly intervals for mortality. Simultaneously, twenty juvenile shrimp were injected with 100 μl of TNE (Tris–HCl–NaCl–EDTA) buffer solution and kept in a 100 L FRP tank. Care was taken to inject these shrimp first before challenging the test animals to avoid contamination. These shrimp served as a control and were kept under
constant observation until the actual challenge experiment was completed. Each family was challenged on separate occasions. Care was taken to maintain uniform conditions for all individuals and families that were challenged. The salinity of the water, the weight of shrimp, the viral dose and the distribution of shrimp in baskets were similar for all the families.

Continuous aeration was provided for the experimental and control tanks. The animals were checked for mortality on an hourly basis. Water temperature was recorded on an hourly basis and pH and salinity was recorded once every morning. The water in the experimental and control tanks were exchanged daily (at 50%) when faecal matter and unused feed at the bottom of the tank was siphoned out in the process. Fresh seawater was provided after removing the debris at the bottom. The cleaning process was carried out daily until the last shrimp died.

When the challenged shrimp started dying, survival data (time to death) along with sex and wet weight of each shrimp were recorded. The dead shrimp were removed and stored at −80°C for DNA extraction.

SNP markers and genotyping
Parents, along with the most susceptible and resistant 40 percentiles of progeny (based on hours of survival post-WSSV infection), were selected from each family for genotyping to find QTL. In all, 1024 offspring belonging to 7 full-sibling families that were challenge tested as described above, were successfully genotyped. Genomic DNA was extracted from the challenged shrimps using the Phenol Chloroform method as described by [83] with slight modifications. The quality of extracted DNA was checked on 2% agarose gel in 1X TBE buffer after electrophoresing at 50 V for an hour. The purity of DNA was checked using OD values at 260 and 280 nm. Quantification was achieved using OD value at 260 nm in Nanodrop 2000C (Thermo Scientific). The DNA of the experimental shrimp was extracted, dissolved in TE (Tris-EDTA) buffer, stored carefully in eppendorf tubes and transported in dry ice to Nofima, Norway for genotyping.

Genotyping was performed with 6 K custom developed Illumina Infinium iSelect Beadchips containing 6 K SNPs from P. monodon transcribed genes [39]. The SNPs were identified by two numbers separated by an underscore, where the first number identified the contig containing the SNP, and the second number was the SNP position in base numbers along the contig length. The same set of SNP genotypes and families used to detect QTL in this paper were previously used to construct a linkage map for P. monodon [39]. The sex averaged map consisted of 3961 informative SNPs which were assigned to 44 linkage groups. We used the map distances for the SNPs on the sex averaged map for the QTL analysis described below. The parentage of the challenge tested animals was checked when the linkage map was created [39].

Genetic parameters, significance of fixed effects and correlation of traits
An animal model was applied to estimate genetic parameters (without accounting for SNP genotype). The animal model decomposed the phenotypic variance into additive genetic and environmental components. Our main interest was whether sex and/or time of challenge (family) should be included as fixed effects in the QTL analysis and whether sex should be included as a covariate. A Markov chain Monte Carlo (MCMC) method using a multi-trait generalised linear mixed effect model (glmm) in a Bayesian estimation framework, with animal breeding value and ID fitted as a random effects, was used for the analysis (R Package, MCMCglmm, [84], http://www.cran.r-project.org). The ID was the same as the animal factor, but was used by MCMCglmm to dissociate individual records from the pedigree and give an indication of between individual variance [85]. The model fitted was,

\[
y = \mu + \text{sex} + \text{family} + \text{animal} + \text{ID}
\]

where \(y\) was time to death, sex and family were fitted as fixed effects, animal and ID were random animal effects and \(\mu\) represented unknown random residual effects. A bivariate model (similar to the above) was used to obtain covariance components, and the genetic correlation between weight and time to death was estimated as,

\[
rg = \frac{\sigma_{A1A2}}{\sqrt{\sigma_{A1}^2 \sigma_{A2}^2}}
\]

where \(\sigma_{A1A2}\) is the estimated additive genetic covariance component between the two traits.

The model was run using 300,000 iterations as burn-in, 1 million iterations for sampling and a thinning interval of 500. A “plausible” prior assuming weak genetic control (additive genetic variance, permanent environmental variance and residual variance accounting for 0.2, 0.1 and 0.7) was used with the smallest possible degree of belief parameter (\(n = 1\)).

Linkage disequilibrium
Linkage disequilibrium measured by \(r^2\) was calculated for all adjacent SNP pairs with the PLINK software package (Purcell et al., 2007).

QTL for WSSV resistance – linkage analysis
Data were analysed using a regression-interval mapping method available through the web-based software GridQTL [86]. The sib-pair model was utilised in order to take
advantage of the full-sib nature of the animal pedigree. Sex was included as a fixed effect, and weight included as a covariate in the model. P-values were calculated for all trait-by-LG combinations with the significance of the peak F-statistic (putative QTL) estimated after 10,000 chromosome-wide permutation tests. A QTL was found to be genome-wide significant if the chromosome-wide significance level was smaller than 0.0011 (0.05/44), a Bonferroni correction based on the number of linkage groups in *P. monodon*. This correction was equivalent to a Benjamini Hochberg [87] false discovery rate of >95% (q-value of 0.98), such that it was expected that more than 95% of the significant results actually were false positives. QTL were denoted as “suggestive” when $P < 0.01$ (before Bonferroni correction).

**QTL for WSSV resistance - GWAS**

QTL GWAS analyses were performed in several ways. First we determined which markers and individuals should be excluded from the GWAS analysis using the check.marker function in GenABEL (www.genabel.org). This function was used to exclude individuals or markers with call rate <95%, markers with minor allele frequency <0.24%, individuals with high autosomal heterozygosity (FDR <1%) and individuals with identity by state ≥0.95. Genomic kingship was computed between all pairs of individuals. We performed a pedigree based association analysis where the pedigree is a confounder (where the heritable trait is more similar between close relatives and therefore some degree of association is expected between any genetic marker and any heritable trait). The effect of the confounding pedigree is expected to inflate the resulting null distribution of the chi square test statistic by a certain constant, lambda. Lambda is a function of the traits heritability and pedigree structure (expressed as a kinship matrix). Two fast tests for genome wide association were applied, Family-based Score Test for Association (FASTA, [88]) and Genome-wide Rapid Analysis using Mixed Models And Score test (GRAMMAS, [42]) using the R package GenABEL. A mixed polygenic model of inheritance was assumed in order to study association in our genetically homogeneous families where *hours of survival* ($y$) was modelled as

$$ y = \mu + G + e $$

where $\mu$ was the intercept, $G$ describes the polygenetic effect (contribution from multiple independently segregating genes all having a small additive effect on the trait) and $e$ describes the random residual effects. The joint distribution of residuals in the pedigree was modelled using a multivariate normal distribution with variance-covariance matrix proportional to the identity matrix. A genomic kingship matrix, generated by calculating the average identity-by-state between individuals in the pedigree (ibs in GenABEL), was used as the relationship matrix for FASTA and GRAMMAS. Both FASTA and GRAMMAS exploit maximum likelihood estimates of the intercept from the polygenic model. One thousand permutations were used to estimate genome wide significance for both the FASTA and GRAMMAS tests. The $P$-value for the 1 degrees of freedom test was corrected for the inflation factor. Genomic control was applied by dividing the observed test statistic ($P$-value for the 1 degrees of freedom test) by the genomic inflation factor $\lambda$ (where $\lambda$ is the regression coefficient of the observed $\chi^2$ test statistic onto the expected $\chi^2$ test statistic). Genomic control is believed by some authors to circumvent the need for Bonferroni correction for multiple testing [89].

The QFAM analysis module in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) was used to perform a linear regression of phenotype on genotype. In this case the module used an adaptive permutation procedure to correct for family structure. Association testing was performed across the total data. Data from a total of 1024 offspring and 14 parents (7 nuclear families) were used with a genotyping success rate of 99%. Minimum number of permutations per SNP was 5, maximum 1 million, alpha level threshold 0, confidence interval on empirical p-value 0.0001 and intercept and slope of the pruning interval 1 and 0.001 respectively. GWAS associations with significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ levels after Bonferroni correction based on the number of linkage groups (which was 44 for *P. monodon*) were noted for all tests. GWAS associations were denoted as “suggestive” when $P < 0.01$ (before Bonferroni correction). As explained for the linkage analysis, the Bonferroni correction was equivalent to a Benjamini Hochberg [87] false discovery rate of >95% (q-value of 0.98).

**Mapping the sex-determining locus**

SNPs significantly associated with sex were detected using a simple $\chi^2$ test of observed and expected allele frequencies in male and female offspring across families under the null hypothesis that the segregation of alleles would be independent of sex. Associations were treated as significant when $P < 0.01$ after Bonferroni correction based on the number of linkage groups. Regression interval mapping using the sib-pair module was also carried out in GridQTL as described for the WSSV analysis using sex as a phenotype.

**Availability of supporting data**

The supporting high density *P. monodon* linkage map and SNP characterisations can be found in [39]. Annotated transcriptome sequence data is available through the Transcriptome Shotgun Assembly Database of NCBI (accession numbers JR196815 – JR235449, http://www.ncbi.nlm.nih.gov/Genbank). Other supporting data (map
position and annotation for linkage mapped transcripts, tests for association with sex) are included in the additional files section.

Additional files

Additional file 1: Map position and annotation for 3961 transcripts
linkage mapped by [39], LG, linkage group. cM, position of SNP on
linkage group in centimorgans. GeneID, closest homology to contig
from BLAST. Length, length of contig in number of bases. NumHits,
number of BLAST matches above threshold (Karlin-Altschul cut off E-score
of 0.001, maximum number of 20). MeltEValue, Karlin-Altschul E-score.

Additional file 2: Map position and tests for association with sex
for transcribed SNPs on LG30. LG, linkage group. cM, position of SNP
on linkage group in centimorgans. GeneID, closest homology to contig
from BLAST. df, degrees of freedom. **, P < 0.01 after Bonferroni
correction. ***, P < 0.001 after Bonferroni correction.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
NR, MB, GG, KVK, TG and AGP designed the research; NR, MB, GG, KVK, MSS,
JS, SJ, CG and PR performed the research; NR and MB contributed new
reagents/analytical tools; NR and MB analysed data; and NR, MB, GG, TG and
KVK wrote the paper. All authors read and approved the final manuscript.

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