The genomic architecture of resistance to Campylobacter jejuni intestinal colonisation in chickens

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Abstract

Background: Campylobacter is the leading cause of foodborne diarrhoeal illness in humans and is mostly acquired from consumption or handling of contaminated poultry meat. In the absence of effective licensed vaccines and inhibitors, selection for chickens with increased resistance to Campylobacter could potentially reduce its subsequent entry into the food chain. Campylobacter intestinal colonisation levels are influenced by the host genetics of the chicken. In the present study, two chicken populations were used to investigate the genetic architecture of avian resistance to colonisation: (i) a back-cross of two White Leghorn derived inbred lines [(61 x N) x N] known to differ in resistance to Campylobacter colonisation and (ii) a 9th generation advanced intercross (61 x N) line.

Results: The level of colonisation with Campylobacter jejuni following experimental infection was found to be a quantitative trait. A back-cross experiment using 1,243 fully informative single nucleotide polymorphism (SNP) markers revealed quantitative trait loci (QTL) on chromosomes 7, 11 and 14. In the advanced intercross line study, the location of the QTL on chromosome 14 was confirmed and refined and two new QTLs were identified located on chromosomes 4 and 16. Pathway and re-sequencing data analysis of the genes located in the QTL candidate regions identified potential pathways, networks and candidate resistance genes. Finally, gene expression analyses were performed for some of the candidate resistance genes to support the results.

Conclusion: Campylobacter resistance in chickens is a complex trait, possibly involving the Major Histocompatibility Complex, innate and adaptive immune responses, cadherins and other factors. Two of the QTLs for Campylobacter resistance are co-located with Salmonella resistance loci, indicating that it may be possible to breed simultaneously for enhanced resistance to both zoonoses.

Keywords: Campylobacter, Chicken, Resistance, Back-cross, Advanced intercross, Genome-wide association, Quantitative trait

Background

Campylobacter is the leading cause of foodborne acute enteritis in humans in the developed world. The condition is usually self-limiting and symptoms last for 5–7 days, but in some cases the infection may be complicated by severe sequelae [1–3]. Epidemiologically implicates poultry as a key reservoir of human infection and up to 80 % of human cases may be attributable to the avian reservoir as a whole [4]. A recent year-long survey found Campylobacter in 73 % of chicken on retail sale in the United Kingdom [5]. There were 66,575 laboratory-confirmed human infections (mostly due to Campylobacter jejuni) and an estimated total of 685,000 cases in the UK in 2013 [6]. The number of Campylobacter jejuni (C. jejuni) in the caeca of chickens can exceed 10^8 colony-forming units (CFU)/g and escape of gut contents and cross-contamination at slaughter is difficult to avoid. Quantitative risk assessments predict that even a relatively modest 2 log_{10} reduction in the number of Campylobacter in broiler
carcasses could reduce the incidence of human disease due to infected chicken by up to 30-fold [7]. Therefore, a pressing need exists for strategies to reduce the entry of *Campylobacter* into the food chain. In the absence of effective licensed vaccines and inhibitors, selection for chickens with increased resistance to *Campylobacter* intestinal colonisation provides a sustainable complimentary control strategy.

There is a widely-held perception that *Campylobacter* is an inert commensal of birds. However, experimental infection of chickens with *Campylobacter* induces a rapid influx of heterophils (the avian functional equivalent of the mammalian neutrophil) into the gut and the production of pro-inflammatory cytokines and chemokines in the intestinal epithelium [8]. Maternal *C. jejuni*-specific antibodies protect chicks against experimental infection [9, 10] and are associated with the delayed incursion of *Campylobacter* into flocks [10, 11]. In some breeds of chicken, *C. jejuni* elicits prolonged inflammatory responses, damage to the intestinal mucosa, diarrhoea and failure to thrive [12, 13]. Conversely, both innate and acquired immune responses have been associated with differential resistance to *Campylobacter* intestinal colonisation [14–16].

The innate immune response to pathogen challenge and disease resistance varies between birds in inbred lines and outbred populations [12, 17–21]. The ability of *C. jejuni* to colonise the intestines differs amongst White Leghorn chicken inbred lines, with lines 6 and N being at the extremes of phenotype [22]. An initial reciprocal backcross experiment between inbred lines 6 and N revealed that the difference in bacterial numbers was heritable [22], but the host genetic mechanism of resistance to *Campylobacter* colonisation is not known. One published genome-wide association study (GWAS) of *C. jejuni* intestinal colonisation status (phenotypes analysed as a binary trait) in a novel dual-purpose chicken breed revealed one candidate locus on chromosome 11 near the *CDH13* gene [23]. There are also several studies of caecal gene expression analysis in chicken lines with different susceptibility to *Campylobacter* colonisation showing variation in transcription of genes influencing immune response [14, 15, 24].

The aim of the present study was to extend a previous investigation of inbred lines of chickens to determine the genetic architecture of resistance to *C. jejuni* colonisation using a focussed genotyping platform. This initially involved challenge of a backcross population (*n* = 288) of White Leghorn chicken inbred lines 6 and N with *C. jejuni* and genotyping of the birds for 1,243 fully informative single nucleotide polymorphism (SNP) markers. An independent replication study was then performed by challenging a 9th generation advanced intercross line (AIL) population (*n* = 218) from a cross of the same two inbred lines with the same *C. jejuni* strain and genotyping with a 580 K SNP high density whole genome DNA array (Affymetrix® Axiom® HD) [25] to refine and identify new quantitative trait loci (QTLs). SNP markers significantly associated with *Campylobacter* intestinal colonisation resistance were detected on chromosomes 4, 7, 11, 14 and 16. We also performed pathway analysis and examined gene expression and re-sequencing data to identify candidate genes within the relevant genomic intervals.

**Results**

**Phenotypes for parental lines, back-cross and AIL birds**

Mean values and standard deviations of log-transformed caecal *C. jejuni* levels following experimental inoculation with strain 11168H for line 6 and N parental birds, the [(6 x N) x N] back-cross and the 9th generation AIL (6 x N) birds are listed in Additional file 1: Table S1. With a single exception of low counts on third day post-infection (dpi), no *C. jejuni* colonies were detected by direct plating of homogenates in any bird of resistant line 6. In contrast, significant levels of *C. jejuni* colonisation were identified in susceptible line N birds, with the number of birds showing colonisation rising over time after infection. The results are consistent with the original report [22]. Resistance was semi-dominant in that levels of *C. jejuni* in the backcross and AIL population, measured five dpi, were intermediate between the levels seen in the two parental lines.

**Interval mapping and GWAS analysis of the back-cross experiment**

The back-cross genotypes were analysed both using interval mapping (linkage analysis) and GWAS analysis (linkage disequilibrium (LD) analysis). In contrast to earlier studies [22, 23] we found with both analyses that levels of bacteria measured in challenged birds behaved as a quantitative trait, and mapped genetically to multiple loci. Two QTLs were detected on chromosomes 7 and 14 that were significantly associated with the log-transformed number of *C. jejuni* in the caeca at 5 dpi by the interval mapping analysis. The QTL on chromosome 7 was located at 26 Mb (Fig. 1) with a 1-LOD interval of 19.3 to 27.12 Mb. This QTL was significant at the chromosome-wide level (*P*-value <0.01) and, with an F value of 12.79, was close to genome-wide significance (5 % F-statistic threshold = 14.73). The QTL on chromosome 14 at 7 Mb (1 LOD interval 2.46 to 13.25 Mb) was significant at the chromosome-wide level (*P*-value <0.05) with an F value of 7.59 (Fig. 1).

GWAS analysis, using the limited informative marker set, identified both significant associations on chromosomes 7 and 14 as in the interval mapping analysis (Table 1). In addition, one SNP on chromosome 11 was
also significantly associated at chromosome-wide level with the log-transformed C. jejuni load (Table 1). The significant SNPs located on chromosome 7 were in high LD with each other ($r^2 = 0.89–0.98$) and the same was the case for the SNPs on chromosome 14 ($r^2 = 0.97$).

Additional file 2: Figure S1 shows the Manhattan plot and the Q-Q plot displaying the GWAS results.

GWAS analyses of the AIL experiment
Multidimensional scaling analysis (MSA) revealed five substructure clusters in the AIL population, which were subsequently included in the GWAS model to correct results for population stratification.

Table 1 List of SNPs associated with log-transformed caecal Campylobacter load at 5dpi in the back-cross population

| SNP name        | Chr | Position (bp) | P-value   | -log$_{10}$ P |
|-----------------|-----|---------------|-----------|---------------|
| Gga_rs15865889  | 7   | 25741058      | 4.2 × 10$^{-3}$ | 3.38          |
| Gga_rs14618024  | 7   | 26003071      | 4.9 × 10$^{-4}$ | 3.31          |
| Gga_rs16597361  | 7   | 24812369      | 6.8 × 10$^{-4}$ | 3.17          |
| Gga_rs15010208  | 14  | 8288336       | 1.3 × 10$^{-3}$ | 2.86          |
| Gga_rs14076550  | 14  | 8716372       | 2.1 × 10$^{-3}$ | 2.68          |
| Gga_snps142-64-19874-S-1 | 11 | 11791311 | 4.2 × 10$^{-3}$ | 2.37          |

SNPs highlighted bold were significant at suggestive genome-wide level ($P < 8.24 × 10^{-4}$) after Bonferroni correction.

GWAS analysis identified two SNPs significantly associated with the log-transformed number of C. jejuni in the caeca at 5 dpi on chromosome 14, located within the 1 LOD interval of the chromosome 14 QTL identified in the back-cross experiment (Table 2). Thus, the QTL on chromosome 14 was confirmed. Additionally, two SNPs crossing the suggestive genome-wide significant threshold were identified on chromosomes 4 and one SNP reaching the chromosome-wide significant threshold on...
chromosome 16 (Table 2). The Manhattan plot and the Q-Q plot for the GWAS results are displayed in Fig. 2.

The estimated additive effects for SNP markers on chromosomes 14, 4 and 16 were $\log_{10} \text{cfu/g} = 1.2$ ($P$-value = 0.01), 1.1 ($P$-value = 0.04) and 2.6 ($P$-value = 0.0001), respectively; proportions of the total phenotypic variance explained were 10, 9 and 6 %, respectively. Collectively, these three loci explained 25 % of the phenotypic variance.

The GWAS data was also reanalysed as a binary trait. This approach identified both the chromosome 4 and 16 significant associations with Campylobacter colonisation status (Table 2) and the significant SNP on chromosome 4 crossed the genome-wide significance threshold (Table 2). The Manhattan plot and the Q-Q plot for the GWAS results from the case-control analysis are displayed in Additional file 3: Figure S2.

**Annotation of QTL regions identified from the back-cross and AIL experiments**

The large region encompassed by the QTL on chromosome 7 contains a relatively small number of genes, a total of 124 genes and 12 microRNA, inside the 1 LOD interval region (Additional file 4: Table S2).

The significant SNP on chromosome 11 is located in an intergenic region between two cadherin genes, cadherin 11 precursor (CDH11) and cadherin 8 (CDH8). Very close to this SNP a third cadherin gene, cadherin 5 (CDH5) was located (Additional file 4: Table S2).

The two significant SNPs identified on chromosome 14 in the AIL experiment were in strong LD and belonged to the same small LD block (154 bp) (Additional file 5: Figure S3). The two significant SNPs were in high LD with the other SNP markers located in regions 0.2 Mb upstream and downstream (Additional file 5: Figure S3). In this 0.4 Mb region, 22 genes and two microRNAs are located (Additional file 4: Table S2). The two significant markers were located in the intronic region of an undescribed gene in the chicken genome. Further investigation in the Ensembl database suggested that this was the orthologue of the N-acetyltransferase 15 (NAT15) gene in humans.

The significant SNPs on chromosome 4 were in high LD and located in a very small LD block (Additional file 6: Figure S4). These SNPs were also in high LD with SNP markers located in regions 0.2 Mb upstream and downstream (Additional file 6: Figure S4). In this 0.4 Mb region, five annotated genes and two microRNAs are located (Additional file 4: Table S2); all significant SNPs were located in the intronic region of the Ephrin receptor A5 (EPHA5) gene.

The significant SNP on chromosome 16 was located inside a single LD block with a length of 224 Kb and was in high LD with the other SNPs located there.

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**Fig. 2** Manhattan plot and Q-Q plot displaying the GWAS results from the AIL experiment (continuous phenotypes). Genomic location is plotted against $-\log_{10}(P)$ in the Manhattan plot (above). Genome-wide ($P < 0.05$) and suggestive genome-wide thresholds are shown as dashed lines. Q-Q plot (below) of observed $P$-values against the expected $P$-values for Campylobacter gut colonisation (log-transformed number of C. jejuni per gram of caecal contents).
Re-sequencing data analysis of back-cross and AIL results

To identify possible protein-coding genes associated with the detected QTLs, the genomic sequences of lines 61 and N birds in the regions of interest were compared. The focus was on the identification of exonic single nucleotide variants (SNVs) with high importance (i.e., non-sense (stop-gain) and missense (non-synonymous) exonic and splicing), since these can affect the function of the gene leading to different isoforms of the transcribed proteins. Genomic regions located within 1 kb upstream of the respective genes were also analysed to identify SNVs with a potential regulatory effect. Due to the lack of regulatory element annotation in chickens, we developed our own pipeline for identifying possible regulatory site mutations. The 1 kb upstream genomic regions were scanned for putative TATA box and CpG island motifs. TATA boxes help facilitate transcription factor binding [26] thus TATA box mutations can effect transcription rates. CpG islands play an important role in methylation regulatory pathways [27]. CpG islands are characterised by regions which have a high density of CpG sites that can be methylated to down-regulate gene expression. Thus mutations in CpG sites can alter transcription regulation.

Summary statistics of all the SNVs identified in the candidate regions for Campylobacter colonisation resistance and all the SNVs detected, the Variant Effect Predictor annotation and the SIFT predictions are presented in Additional file 8: Figure S6 and Additional file 9: Table S3, respectively. In total, 20,125 variants were identified. The SNVs located in exonic regions were in total less than 5 % while the rest of the SNVs (95 %) were located in intronic, upstream and downstream regions. A few genes with SNVs that potentially could lead to non-functional transcripts were detected. More specifically, two genes AXIN 1 located on chromosome 14 and BG1 on chromosome 16 were found to contain a stop-gain SNV, a sequence variant whereby at least one base of a codon is changed, resulting in a premature stop codon, a leading to a shortened transcript; three genes, BG1 and ENSGALG00000028367 located on chromosome 16 and CCDC108 on chromosome 7 contained a splice acceptor variant, a splice variant that changes the 2 base region at the 3’ end of an intron and might lead to splicing changes; two genes, RACGAPI and SPEG on chromosome 7 contained a splice donor variant, a splice variant that changes the 2 base region at the 5’ end of an intron and can lead to splicing changes, as well. Furthermore, seventeen genes contained missense and according to SIFT prediction deleterious SNVs that might create partially or completely non-functional proteins. More specifically, C16orf96 gene on chromosome 14, B42, B4FIV21, B-BTN2, TAP2 and ILAII on chromosome 16, IFIHI, LY75, SLC11A1, SLC38A11, SPEG, ZNF142, CCDC108, TT2C21B, OBSLI, PTPRN, GLEBL1 on chromosome 7 had missense deleterious SNVs. Several other genes contained SNVs with moderate impact. Details of genes containing splicing, 5' UTR, both missense and UTR SNVs are presented in Additional file 10: Table S4.

TATA box motifs were identified in the upstream region of some genes but no variation was detected there. On the other hand, CpG island motifs were detected in many of the genes studied and some had SNVs. However, only in few cases the SNVs occur in CpG sites. Details of the genes containing SNVs in CpG sites are presented in the Additional file 10: Table S4.

The gene transcript from each experiment with the highest rate of non-synonymous coding SNVs (i.e., number of non-synonymous SNVs divided by the length of the coding DNA sequence (CDS) of the transcript, dN/L), rate of non-synonymous to synonymous SNVs (dN/dS), rate of exonic SNVs (number of exonic SNVs divided by exonic length of the transcript), rate of intronic SNVs (number of intronic SNVs divided by intronic length of the transcript) are presented in Table 3. These rates were considered to pertain to transcripts that differed between the two parental lines and an indication of positive selection that might result in the creation of different alleles responsible for functional differences in immune responses affecting disease resistance in the two lines.

Ingenuity pathway analysis of back-cross and AIL results

To identify potential canonical pathways and networks underlying the QTLs detected, we performed pathway analysis using the genes located in these regions. Pathways involved in innate and adaptive immune response, inflammatory response, response to infectious diseases, cell signalling and adhesion, and metabolism constituted the majority of the pathways highlighted for both back-cross and AIL results (Fig. 3). Moreover, two networks of molecular interactions related to immune response were constructed using the list of candidate genes for AIL (Additional file 11: Figure S7).

Gene expression analysis

Many quantitative traits are associated with altered gene expression rather than coding variation. For example, variable expression of the satiety signal receptor, CCKAR, is associated with appetite control in chickens [28]. Two chemokine genes, CXCL1i1 and CXCL1i2, lie in close proximity with the significant markers identified on chromosome 4. Both chemokines are induced after
| Gene          | Transcript     | Chr | dN/dS | dN/L  | exon_rate | intron_rate |
|--------------|----------------|-----|-------|-------|-----------|-------------|
| A            | ENSGALG00000026721 | 7   | 3     | 0.002441 | 0.003247 | No introns  |
|              | FAM134A        | 7   | 3     | 0.002098 | 0.002797 | 0.006759    |
|              | RALB           | 7   | 3     | 0.004342 | 0.00519  | 0.00601     |
|              | IFI1           | 7   | 2     | 0.000608 | 0.00743  | 0.000568    |
|              | ARPC2          | 7   | 2     | 0.001768 | 0.002418 | 0.003158    |
|              | PECR           | 7   | 2     | 0.002235 | 0.003007 | 0.006175    |
|              | LZTR1          | 7   | 2     | 0.002436 | 0.003925 | 0.003483    |
|              | NIFK           | 7   | 2     | 0.002424 | 0.002251 | 0.006897    |
|              | CCDC14         | 7   | 2     | 0.000729 | 0.01017  | 0.004697    |
|              | TIC21B         | 7   | 1.75  | 0.001702 | 0.002346 | 0.004126    |
|              | SMARCAL1       | 7   | 1.333333 | 0.001388 | 0.004471 | 0.005288    |
|              | C16orf96       | 7   | 1.25  | 0.008818 | 0.014139 | 0.00646     |
|              | COBL1          | 7   | 1.2   | 0.001736 | 0.003179 | 0.003251    |
|              | OBSL1          | 7   | 1.142857 | 0.002852 | 0.00482  | 0.009001    |
|              | SLC38A11       | 7   | 1     | 0.000749 | 0.003485 | 0.007136    |
|              | FIGN           | 7   | 1     | 0.000446 | 0.000892 | No introns  |
|              | FAP            | 7   | 1     | 0.000444 | 0.001297 | 0.002828    |
|              | ASIC4          | 7   | 1     | 0.001254 | 0.002503 | 0.008669    |
|              | ZFAND2B        | 7   | 1     | 0.002137 | 0.004343 | 0.007585    |
|              | SLC11A1        | 7   | 1     | 0.00119  | 0.002381 | 0.009314    |
|              | C16orf96       | 7   | 1.25  | 0.008818 | 0.014139 | 0.00646     |
|              | ENSGALG00000023695 | 14  | 1     | 0.000587 | 0.00534  | 0.002789    |
|              | RALB           | 7   | 3     | 0.004342 | 0.00519  | 0.00601     |
|              | GLD3           | 7   | NA    | 0.003421 | 0.003421 | 0.00852     |
|              | OBSL1          | 7   | 1.142857 | 0.002852 | 0.00482  | 0.009001    |
|              | ENSGALG00000026721 | 7   | 3     | 0.002441 | 0.003246 | 0.004126    |
|              | LZTR1          | 7   | 2     | 0.002436 | 0.003925 | 0.003483    |
|              | NIFK           | 7   | 2     | 0.002424 | 0.002251 | 0.006897    |
|              | ENSGALG00000023707 | 14  | 0.285714 | 0.002304 | 0.010333 | 0.008303    |
|              | CCDC108        | 7   | 0.75  | 0.002268 | 0.005093 | 0.009366    |
|              | PECR           | 7   | 2     | 0.002235 | 0.003007 | 0.006175    |
|              | TMEM169        | 7   | 1     | 0.002176 | 0.003236 | 0.007113    |
|              | ZFAND2B        | 7   | 1     | 0.002137 | 0.004343 | 0.007585    |
|              | FAM134A        | 7   | 3     | 0.002098 | 0.002797 | 0.006759    |
|              | CYP27A1        | 7   | 0.75  | 0.001911 | 0.004882 | 0.003762    |
|              | RACGAP1        | 7   | 0.6   | 0.001808 | 0.004455 | 0.007782    |
|              | NHEJ1          | 7   | 1     | 0.001795 | 0.011236 | 0.006046    |
|              | ARPC2          | 7   | 2     | 0.001768 | 0.002418 | 0.003158    |
|              | SLC4          | 7   | 1     | 0.001744 | 0.004729 | 0.005968    |
|              | COBL1          | 7   | 1.2   | 0.001736 | 0.003179 | 0.003251    |
|              | C16orf96       | 7   | 1.25  | 0.008818 | 0.014139 | 0.00646     |
| Gene    | Transcript | Chr | dN/dS | dN/L | exon rate | intron rate |
|---------|------------|-----|-------|------|-----------|-------------|
| MNR2    | ENSGALT0000018518 | 7   | 0     | 0    | 0.012723  | 0.00565     |
| NHEJ1   | ENSGALT0000018498 | 7   | 0     | 0    | 0.011236  | 0.005646    |
| TUBA4A  | ENSGALT0000018477 | 7   | 0     | 0    | 0.010549  | 0.004069    |
| ENSGALG000000023707 | ENSGALT00000039621 | 14  | 0.285714 | 0.002304 | 0.010333  | 0.008303    |
| TUBA4A  | ENSGALT00000038318 | 7   | 0.058824 | 0.000729 | 0.010204  | 0.017134    |
| HSPBAP1 | ENSGALT0000019085 | 7   | 0.25  | 0.001374 | 0.006512  | 0.006158    |
| C2orf62 | ENSGALT0000029291 | 7   | 0.166677 | 0.000938 | 0.006416  | 0.008264    |
| ABCB6   | ENSGALT0000038272 | 7   | 0.2   | 0.000805 | 0.006006  | 0.007832    |
| ERCC3   | ENSGALT00000018775 | 7   | 0     | 0    | 0.005964  | 0.01652     |
| PLA2R1  | ENSGALT00000033111 | 7   | 0.5   | 0.00138 | 0.005762  | 0.008359    |
| STK16   | ENSGALT00000018480 | 7   | 0     | 0    | 0.005722  | 0.006939    |
| ENSGALG000000023695 | ENSGALT00000039593 | 14  | NA    | 0.005587 | 0.00534   | 0.002789    |
| GLB1L   | ENSGALT00000018482 | 7   | 0.5   | 0.001586 | 0.005227  | 0.012599    |
| RALB    | ENSGALT00000018997 | 7   | 3     | 0.004342 | 0.00519   | 0.00601     |
| CCDC108 | ENSGALT0000018523 | 7   | 0.75  | 0.002268 | 0.005093  | 0.009366    |
| CYP27A1 | ENSGALT0000003899 | 7   | 0.75  | 0.001911 | 0.004882  | 0.003762    |
| EPB41L5 | ENSGALT0000018952 | 7   | 0     | 0    | 0.004822  | 0.006925    |
| OBSL1   | ENSGALT00000018337 | 7   | 1.142857 | 0.002852 | 0.00482   | 0.009001    |
| SPEG    | ENSGALT00000043042 | 7   | 0.194444 | 0.000763 | 0.004805  | 0.005843    |
| TUBA4A  | ENSGALT00000038318 | 7   | 0.058824 | 0.000729 | 0.010204  | 0.017134    |
| ERCC3   | ENSGALT00000018775 | 7   | 0     | 0    | 0.005964  | 0.012599    |
| GLB1L   | ENSGALT00000018482 | 7   | 0.5   | 0.001586 | 0.005227  | 0.012599    |
| RUFY4   | ENSGALT00000043062 | 7   | 0.333333 | 0.001133 | 0.00315   | 0.01241     |
| ATG9A   | ENSGALT00000019038 | 7   | 0     | 0    | 0.002891  | 0.011987    |
| RPL37A  | ENSGALT00000018702 | 7   | 0     | 0    | 0         | 0.011268    |
| C16orf5 | ENSGALT00000012346 | 14  | 0     | 0    | 0.002632  | 0.011263    |
| AAMP    | ENSGALT00000018663 | 7   | 0     | 0    | 0.001614  | 0.010427    |
| DES     | ENSGALT00000018446 | 7   | 0     | 0    | 0.003731  | 0.010335    |
| CTDSP1  | ENSGALT00000045561 | 7   | 0     | 0    | 0.001002  | 0.010093    |
| ANKZF1  | ENSGALT00000018493 | 7   | 0     | 0    | 0.004431  | 0.010028    |
| IGBP2   | ENSGALT00000018698 | 7   | 0     | 0    | 0.003279  | 0.009894    |
| CCDC108 | ENSGALT00000018523 | 7   | 0.75  | 0.002268 | 0.005093  | 0.009366    |
| SLC23A3 | ENSGALT00000018510 | 7   | 1     | 0.00119 | 0.002381  | 0.009314    |
| OBSL1   | ENSGALT00000018337 | 7   | 1.142857 | 0.002852 | 0.00482   | 0.009001    |
| ASIC4   | ENSGALT00000018356 | 7   | 1     | 0.001254 | 0.002503  | 0.008669    |
| LY75    | ENSGALT00000018187 | 7   | 0.266667 | 0.000775 | 0.00358   | 0.008563    |
| PLACL2R1| ENSGALT00000033111 | 7   | 0.5   | 0.000138 | 0.005762  | 0.008359    |
| ENSGALG000000023707 | ENSGALT00000039621 | 14  | 0.285714 | 0.002304 | 0.010333  | 0.008303    |
| C2orf62 | ENSGALT0000029291 | 7   | 0.166677 | 0.000938 | 0.006416  | 0.008264    |
| ARHGEF1 | ENSGALT00000045992 | 7   | 0     | 0    | 0.002606  | 0.008085    |

B

| Gene    | Transcript | Chr | dN/dS | dN/L | exon rate | intron rate |
|---------|------------|-----|-------|------|-----------|-------------|
| BF2     | ENSGALT00000046170 | 16  | 9     | 0.00878 | 0.009381  | 0.010121    |
| BF2     | ENSGALT0000000139  | 16  | 9     | 0.00885 | 0.009452  | 0.01004     |
Table 3 Genes and transcripts located in the QTL candidate regions for *Campylobacter* resistance with the highest variation among the two parental lines. (A) Backcross experiment (B) Advanced Intercross Experiment (Continued)

| Gene     | GenBank Accession | Chromosome | Log2 Fold Change | p-value       | Discounted p-value | raw p-value       | Adjusted p-value |
|----------|-------------------|------------|------------------|---------------|-------------------|-------------------|-----------------|
| BF2      | ENSGALT00000044107| 16         | 9                | 0.00858       | 0.013504          | 0.010373         |
| BF2      | ENSGALT00000043207| 16         | 9                | 0.008515      | 0.013432          | 0.010460         |
| RASSF6   | ENSGALT000000019068| 4          | 2                | 0.001974      | 0.005961          | 0.006711         |
| BLB1     | ENSGALT000000008925| 16         | 2                | 0.002994      | 0.002985          | 0             |
| TAPBP    | ENSGALT00000000203| 16         | 1.66667          | 0.003915      | 0.006672          | 0.007874         |
| C16orf96 | ENSGALT00000042897| 14         | 1                | 0.008818      | 0.014139          | 0.006646         |
| TNXB     | ENSGALT00000000238| 16         | 1.25             | 0.002016      | 0.003629          | 0.001132         |
| SLX4     | ENSGALT00000046089| 14         | 1                | 0.001744      | 0.004729          | 0.005968         |
| TAPBP    | ENSGALT00000044889| 16         | 1                | 0.002132      | 0.003899          | 0.010359         |
| SLX4     | ENSGALT00000012367| 14         | 0.875            | 0.001439      | 0.004358          | 0.006141         |
| TAPBP    | ENSGALT00000044889| 16         | 1                | 0.002132      | 0.003899          | 0.010359         |
| TRIM27   | ENSGALT00000044107| 16         | 0.6              | 0.005976      | 0.016882          | 0.010012         |
| TRAP1    | ENSGALT00000012549| 14         | 0.5              | 0.000286      | 0.008137          | 0.006749         |
| HLADMB   | ENSGALT00000041213| 16         | 0.5              | 0.001789      | 0.005938          | 0.019055         |
| TRIM41   | ENSGALT00000045935| 16         | 0.5              | 0.001744      | 0.004208          | 0.001730         |
| TRIM27   | ENSGALT00000042650| 16         | 0.5              | 0.000892      | 0.002621          | 0.003252         |
| TAP2     | ENSGALT00000000237| 16         | 0.75             | 0.002477      | 0.005783          | 0.013439         |
| TGFB     | ENSGALT00000046170| 16         | 1.25             | 0.003820      | 0.009537          | 0.010099         |
| BF2      | ENSGALT00000012459| 14         | 1                | 0.005976      | 0.016882          | 0.010126         |
| BF2      | ENSGALT00000044107| 16         | 1.66667          | 0.003915      | 0.006672          | 0.007874         |
| BF2      | ENSGALT00000043207| 16         | 1.333333         | 0.00312       | 0.005109          | 0.010619         |
| RASSF6   | ENSGALT00000019068| 4          | 2                | 0.001974      | 0.005961          | 0.006711         |
| BLB1     | ENSGALT000000008925| 16         | 2                | 0.002994      | 0.002985          | 0             |
| TAPBP    | ENSGALT00000000203| 16         | 1.66667          | 0.003915      | 0.006672          | 0.007874         |
| C16orf96 | ENSGALT00000042897| 14         | 1.25             | 0.002016      | 0.003629          | 0.001132         |
| TNXB     | ENSGALT00000000238| 16         | 1.25             | 0.002016      | 0.003629          | 0.001132         |
| RASSF6   | ENSGALT00000019068| 4          | 2                | 0.001974      | 0.005961          | 0.006711         |
| BLB1     | ENSGALT000000008925| 16         | 2                | 0.002994      | 0.002985          | 0             |
| HLADMB   | ENSGALT00000045935| 16         | 0.5              | 0.002865      | 0.008137          | 0.006479         |
| DM2      | ENSGALT00000000222| 16         | 0.4              | 0.002581      | 0.013383          | 0.014400         |
| TAPBP    | ENSGALT00000044889| 16         | 1                | 0.002132      | 0.003899          | 0.010359         |
| TNXB     | ENSGALT00000000238| 16         | 1.25             | 0.002016      | 0.003629          | 0.001132         |
| RASSF6   | ENSGALT00000019068| 4          | 2                | 0.001974      | 0.005961          | 0.006711         |
| BLB1     | ENSGALT000000008925| 16         | 2                | 0.002994      | 0.002985          | 0             |
| HLADMB   | ENSGALT00000045421| 16         | 0.5              | 0.002581      | 0.013383          | 0.014400         |
| DM2      | ENSGALT00000000222| 16         | 0.4              | 0.002581      | 0.013383          | 0.014400         |
| TAPBP    | ENSGALT00000044889| 16         | 1                | 0.002132      | 0.003899          | 0.010359         |
| TNXB     | ENSGALT00000000238| 16         | 1.25             | 0.002016      | 0.003629          | 0.001132         |
| RASSF6   | ENSGALT00000019068| 4          | 2                | 0.001974      | 0.005961          | 0.006711         |
| BLB1     | ENSGALT000000008925| 16         | 2                | 0.002994      | 0.002985          | 0             |
| HLADMB   | ENSGALT00000045421| 16         | 0.5              | 0.002581      | 0.013383          | 0.014400         |
| DM2      | ENSGALT00000000222| 16         | 0.4              | 0.002581      | 0.013383          | 0.014400         |
Campylobacter infection in chickens [8, 12, 29] and might be involved in effective host immune response. We therefore examined their expression in caecal tonsils of challenged birds. The data are shown in Additional file 12: Figure S8. Data are expressed as the fold change in mRNA levels when samples from infected birds were compared to non-infected birds of the same age from each line. In C. jejuni infected birds from both lines CXCL1 and CXCL2 were down-regulated. Nevertheless, the level of chemokine CXCL2 was decreased in line N significantly (P-value <0.05) more than in line 61, while CXCL1 levels did not significantly differ between the lines.

Table 3 Genes and transcripts located in the QTL candidate regions for Campylobacter resistance with the highest variation among the two parental lines. (A) Backcross experiment (B) Advanced Intercross Experiment (Continued)

| Gene  | Ensembl ID   | Chromosome | Length (kb) | dN | dS | dN/L | P-value   |
|-------|--------------|------------|-------------|----|----|------|-----------|
| BNK   | ENSGALT0000001988 | 16 | NA          | 0.013462 | 0.011445 | 0.003878 |
| DMB2  | ENSGALT0000000222 | 16 | 0.4        | 0.002581 | 0.010393 | 0.014409 |
| ZNF692| ENSGALT00000031515 | 16 | 0.454545 | 0.003215 | 0.00949 | 0.01307 |
| BF2   | ENSGALT0000000139 | 16 | 9          | 0.00885 | 0.009452 | 0.00109 |
| BF2   | ENSGALT000000046170 | 16 | 9          | 0.00878 | 0.009381 | 0.010121 |
| HLA DM8| ENSGALT00000041213 | 16 | 0.5        | 0.002865 | 0.008121 | 0.011547 |
| DMB2  | ENSGALT000000041214 | 16 | 0          | 0 | 0.008121 | 0.011547 |
| TAP1  | ENSGALT00000045907 | 16 | 0.230769 | 0.001726 | 0.006978 | 0.010147 |
| TRIM27| ENSGALT00000000172 | 16 | 1.666667 | 0.003915 | 0.006672 | 0.007874 |
| IL41  | ENSGALT00000000109 | 16 | 0.428571 | 0.001912 | 0.005938 | 0.006711 |
| RASSF6| ENSGALT00000019068 | 4 | 2          | 0.001974 | 0.009593 | 0.011905 |
| TCLEC2D| ENSGALT00000000183 | 16 | 0.5        | 0.001789 | 0.009538 | 0.011905 |
| ZNF692| ENSGALT00000031515 | 16 | 0.454545 | 0.003215 | 0.00949 | 0.01307 |
| ZNF692| ENSGALT00000031515 | 16 | 0.454545 | 0.003215 | 0.00949 | 0.01307 |
| ZNF692| ENSGALT00000031515 | 16 | 0.454545 | 0.003215 | 0.00949 | 0.01307 |
| ZNF692| ENSGALT00000031515 | 16 | 0.454545 | 0.003215 | 0.00949 | 0.01307 |
| TAP1  | ENSGALT00000045907 | 16 | 0.230769 | 0.001726 | 0.006978 | 0.010147 |

Non synonymous SNVs/synonymous SNVs(dN/dS); non synonymous SNVs/CDS length (dN/L); NA = no synonymous SNV present; with bold is the rate based on which the genes were ranked.
Selection of candidate genes

A total of 20 genes were selected amongst all genes located in the regions of interest identified from the analysis of the back-cross and AIL populations as good candidate genes for avian resistance to *Campylobacter* colonisation (Additional file 13: Table S5). Gene selection was based on their biological function, proximity to significant markers, sequencing differences, mRNA expression (tested for *CXCLI1* and *CXCLI2*) after *Campylobacter* infection, their involvement in immune response pathways and networks, and any previously known involvement in other infectious diseases in poultry (Additional file 9: Table S4).

Discussion

The present study indicates that the precise level of *Campylobacter* intestinal colonisation is a heritable complex quantitative trait under the genetic control of multiple loci, genes and linked sequence variants. In the back-cross analysis we identified candidate QTLs in three genomic locations on chromosomes 7, 11 and 14. Using a 9th generation AIL population we refined the location of the QTL identified on chromosome 14 (from a 12 Mb region to a 0.4 Mb region) and detected two additional QTLs located on chromosomes 4 and 16. The initial candidate regions identified in the back-cross experiment have limited resolution and the detected QTLs may reflect the effect of many linked variants which are separated when the LD blocks break after many generations of recombination [30]. The identification of new QTLs in the AIL analysis could be attributed to genotyping based on many more markers (approximately 300,000 compared to 1,300 in the back-cross experiment), which considerably increased the power of QTL detection.

Two good candidate genes, TRAP 1 and AXIN 1 were identified in the refined QTL location on chromosome 14; this exemplifies the common findings from the two
experimental designs (back-cross and AIL). TRAPI is coding for a mitochondrial heat shock protein with antioxidant and anti-apoptotic functions [31, 32]. Our resequencing data revealed that TRAPI is divergent between the two lines. It has a high SNV/L and dN/dS rate that may affect its function and also has 3’/5’ UTR SNV that may impact on gene expression. AXIN 1, is coding for a scaffolding protein controlling the levels of β-catenin, which in turn regulates NF-kB activity. This gene is also very divergent in the two lines with a stop/coding SNV, a high dN/L and dN/dS rate as well as UTR SNV.

The two parental lines, 6, and N, have a different MHC haplotype, B2 and B21, respectively. Therefore, the involvement of MHC, a genomic region that encodes molecules that provide the context in which T cells recognize foreign antigens, in Campylobacter colonisation resistance, is not unexpected. This genomic region is the focus of considerable interest because of the strong, reproducible infectious disease associations found with specific MHC haplotypes [33–36]. However, the highly polymorphic nature of the genes, the strong LD that exists within this region [37–39], and the limited number of SNP markers on chromosome 16 present obstacles to associating individual genes with disease responses. The previous study of the inbred lines suggested that MHC was not involved [22], but this was based on only 41 back-cross (between lines 6, and N) birds and the genotyping was performed using only one microsatellite marker located close to the MHC. The study of Boyd et al. inferred autosomal dominance at a single locus, but that conclusion was based upon the failure to detect the differential colonisation between the parent line 6, and crossed birds [22].

As noted in the introduction, the only previous GWAS study of Campylobacter intestinal colonisation resistance in chickens [23], identified a risk locus on chromosome 11 associated with the T-cadherin (CDH13) gene. This finding might be linked to the cadherin genes (CDHS, CDH11, CDH8) identified in our back-cross analysis. Cadherins are a super-family of calcium-dependent proteins, with a significant role in cell-cell adhesion and the maintenance of structural and functional tissue integrity [40]. The cadherins might interact with C. jejuni and consequentially affect resistance to colonisation either by facilitating internalisation or inciting a protective host response [41–44].

Connell et al. examined gene expression of caeca tissue from high colonised and nil colonised birds of the same breed challenged with Campylobacter, and found evidence of more rapid innate immune response to the infection in nil colonised birds [14]. Among the candidates genes identified in the current study, there are two genes associated with two major pro-inflammatory chemokines (CXCLI1 and CXCLI2) that are reportedly induced during Campylobacter infection both in chickens and humans [45, 46]. Stimulated heterophils produce CXCLI1 and CXCLI2 to induce leukocyte (primarily heterophil) chemotaxis [47, 48]. Resequencing data analysis of the two parental inbred lines (6, and N) revealed no protein-coding variation, but did identify an SNV in the 3’UTR of CXCLI2. Although SNV located in the regulatory regions of individual genes are of high importance, relevant knowledge in chickens is very limited and literature virtually non-existent. Whether this particular variant or more distal control elements are responsible, direct measurement of mRNA levels in caecal tonsils of infected birds confirmed differential expression of CXCLI2 between the two lines. Similarly, Connell et al. detected a differential expression only for CXCLI2 [14]. According to these results CXCLI2 may plausibly contribute to resistance to Campylobacter colonisation in chickens.

A previous study of SNP associations with innate and adaptive immune responses in laying hens identified a QTL on chromosome 7 at the same region where the peak of our QTL on the same chromosome is located, associated with complement activity [49]. Several other innate immune genes, very different at sequence level between the two inbred lines, lie under the peak of our identified QTL within the 1 LOD-drop confidence interval. The interferon-induced helicase C domain-containing protein 1 (IFIH1) gene with a predicted splicing SNV is located at 20.5 Mb, the lymphocyte antigen 75 precursor (LY75) gene, also known as CD205, which encodes a receptor on dendritic cells, is located at 21.5 Mb. The natural resistance-associated macrophage protein 1 (NRAMP1) gene, also known as SLC11A1, located at 22 Mb, is another potentially exciting candidate gene to explain this QTL. NRAMP1 regulates intracellular pathogen proliferation and macrophage inflammatory responses, by influencing the phagolysosomal function of macrophages. Variants in this gene have been associated with resistance to Salmonella infection in mice and poultry [50, 51]. Interestingly, host resistance to C. jejuni infection in the mouse is also NRAMP1-dependent [52].

Inbred lines 6, and N are resistant and susceptible respectively not only to Campylobacter gut colonisation, but also to Salmonella gut colonisation, supporting speculation that the genetic control of colonisation could be at least partly common for these bacteria [53]. Extensive QTL mapping studies have taken place to identify loci for resistance to S. enterica serovar Typhimurium and Enteritidis colonisation over the last 20 years using these two inbred chicken lines [2, 20, 51, 54, 55]. Interestingly, two QTLs for resistance to enteric carriage of Salmonella have been identified at the same regions on chromosome 14 [54] and 16 [51, 55] as in our study.
Both TRAP1 and AXIN 1, the two putative candidate genes on chromosome 14 for Campylobacter colonisation resistance, have been associated with Salmonella infection in previous studies [56, 57].

Line 61 is also resistant to infectious bursal disease virus (IBDV) and Marek’s disease virus (MDV) while line N is resistant to infectious bronchitis virus (IBV) and MDV. Whole-genome gene expression analyses comparing resistant line 61 with susceptible inbred line 7 for MDV and inbred Brown Leghorn [Brl] line for IBDV have been conducted to investigate the host response to these infections [58–63]. Likewise, similar analysis comparing resistant line N with susceptible inbred line 15I for IBV has been conducted. Among the genes highlighted as candidate genes for IBV, IBDV or MDV resistance, with different expression during the host response and differential expression between the resistant and susceptible lines, were many of the genes identified in our study as good candidates for Campylobacter intestinal colonisation resistance (Additional file 13: Table S5). These genes may play a central role in an overall effective host immune response.

Conclusion
The multiple QTLs identified as well as the many immune response and inflammation pathways and the predicted involvement of MHC attest to a complex trait controlled by many candidate genes each with a moderate or weak effect. However, the magnitude of the additive effect size of the significant markers and the large proportion of the phenotypic variance explained by them is encouraging for informing breeding strategies for enhanced Campylobacter colonisation resistance in broiler chicken. A study by Gormley et al. revealed that levels of contamination generated by natural exposure varied by at least two orders of magnitude in a wide range of commercial broiler genotypes [64]. As our findings pertain to inbred lines and linkage disequilibrium will likely be different in outbred broiler populations future research is now warranted to explore the genetic basis for resistance in commercial birds and extent of variation at the QTLs identified. Our study highlights the utility of using inbred lines as a resource to map resistance-associated loci, gaining increased power to detect QTLs with modest effects, and informs the design of selective breeding strategies for control of a major zoonosis.

Methods
Ethical statement
All animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986, with the approval of the Ethical Review Committee of The Pirbright Institute (under project licence PPL 30/2462) and the Animal Welfare and Ethical Review Body of The Roslin Institute (under PPL 60/4420).

Animals
Chicken inbred lines 61 (resistant to Campylobacter colonisation) and N (susceptible to Campylobacter colonisation) were originally derived from White Leghorn flocks at the USDA-ARS Avian Disease and Oncology Laboratory in East Lansing, MI, USA. The lines have been maintained by random mating within the specified-pathogen-free (SPF) flocks at the Pirbright Institute in the UK since 1972 (line 61) and 1982 (line N). To generate the back-cross (n = 288 for the present study), F1 progeny (61 x N) were crossed with the susceptible line N.

Colonies of lines 61 and N were given to INRA Tours by the Pirbright Institute over a decade ago. A 9th generation AIL between lines 61 and N was generated there. At each generation, animals from distinct families were crossed to minimise inbreeding and increase the number of recombinations as suggested by Darvasi and Soller [65]. In total, 218 AIL birds were generated for the present study.

Bacterial challenge
In the parent lines, back-cross and AIL, day-old birds were orally inoculated with 0.1 ml of an overnight Mueller-Hinton broth culture of Campylobacter-free gut flora originally taken from the caecal contents of an adult SPF chicken. Birds were orally challenged at three weeks of age with 10⁸ CFU C. jejuni strain 11168H, a hypermotile variant of the sequenced strain NCTC11168 that readily colonises chickens [66] or with control Mueller-Hinton medium only. To confirm resistance or susceptibility to Campylobacter colonisation, control birds (n = 4–5 per line) and infected birds (n = 8–10 per line) were killed at 2, 3, 4 and 5 dpi and a sample of caecal contents was obtained. In addition, caecal tonsil samples of the birds killed 5 dpi were removed to RNAlater for RNA extraction, and gene expression analysis, as described below. Three separate challenge experiments were performed for logistical reasons on the back-cross and AIL populations to assess the level of Campylobacter colonisation in which birds were killed at 5 dpi and caecal contents were removed to measure the Campylobacter load and blood samples were taken for DNA extraction and genotyping, as described below.

Phenotyping
The level of bacterial colonisation in the caecal contents of infected birds was determined in all birds from the back-cross and AIL populations as well as the respective parents as described previously [67]. Bacterial counts for both the back-cross and AIL population were skewed
and were accordingly log-transformed in order to normalise their distribution. Thus, phenotypes were expressed on a continuous scale as log-transformed bacterial counts. Moreover, in the case of AIL the phenotypes were also expressed on a binary (0/1) scale indicating absence or minimal colonisation (i.e., no colonies detected by direct plating of 0.1 g homogenate; thus corresponding to birds with Campylobacter load ≤ 100 CFU/g of content)/presence of colonisation.

Genotyping and QTL detection

This part of the study was conducted as two separate experiments described below.

Back-cross experiment

QTL mapping analysis

Over 18,000 genome-wide SNPs were screened in the parent lines (61 and N) to identify fully informative markers for the back-cross mapping study. All SNPs were available through Ensembl (www.ensembl.org) and were analysed using existing panels of chicken SNPs. Markers were selected in the parent lines on the basis of their fixation for the alternate allele at each position, providing maximum information content in the back-cross population. A total of 1,385 SNPs were selected from the 18,000 SNPs panel for analysis in the present study. Genotyping and quality control were performed as described before [20]. A full list of the SNPs (1,243) used in the final analysis is displayed in Additional file 14: Table S6. Positions of SNP markers were obtained using the Gal-gal4 assembly in Ensembl Genome Browser (www.ensembl.org).

QTL analysis was performed by regression interval mapping [68] using the QTL Express software [69] available through GRIDQTL (http://gridqt1.cap.ed.ac.uk) as described previously [20].

GWAS analysis

The back-cross genotypes were also analysed in a GWAS in order to compare with and validate interval mapping results. The following thresholds were used for quality control: minor allele frequency (MAF) < 0.05 and call rate > 95 %. Deviation from Hardy-Weinberg equilibrium was not considered as a criterion for excluding SNPs since this was a back-cross population. After the quality control, 1,212 SNP markers remained for further analysis. The software GEMMA [70] was used to run the GWAS analysis using a standard univariate linear mixed model in which sex (male, female) and experiment number (1, 2, 3) were fitted as fixed effects and the genomic relationship matrix among individuals was included as a random effect. A Bonferroni correction was applied for multiple testing [71]. After Bonferroni correction, significance thresholds were $P \leq 4.12 \times 10^{-5}$ and $P \leq 8.24 \times 10^{-4}$ for genome-wide ($P \leq 0.05$) and suggestive (namely one false positive per genome scan) levels, corresponding to -log$_{10}$($P$) of 4.38 and 3.08, respectively. Searches for significant SNPs were performed also at chromosome-wide level ($P \leq 0.05$).

SNP validation

Individual markers found to be significant in GWAS were further verified in an association analysis where each SNP was fitted as a fixed effect simultaneously with other fixed effects previously fitted (in the GWAS analysis) in a mixed model. The analysis was carried out with the software ASREML [72]. This analysis yielded estimates of the magnitude of the additive SNP effect, as the difference in value between the homozygous (line N-AA) and the heterozygous (AB) genotypes. Solutions were the predicted trait values for each genotype class.

Finally, to evaluate the extent of LD and identify potential regions of causal mutations for Campylobacter colonisation resistance, LD among SNPs was calculated as an r-square value using the software Plink [73]. Furthermore, LD blocks in the regions where significant SNPs were found with GWAS were visualised using the software Haplovie[74].

Advanced intercross line experiment

GWAS

AIL birds were genotyped using a 580 K SNP high density whole genome SNP array (Affymetrix® Axiom® HD) [74]. The SNP genotype data were subjected to quality control measures using the following thresholds: MAF < 0.02 and call rate < 95 %. Deviation from Hardy-Weinberg equilibrium was not considered as a method for excluding SNPs since this was an AIL population. After quality control, 286,432 SNP markers remained for further analysis. Positions of SNP markers were obtained using the Gal-gal4 assembly in Ensembl Genome Browser (www.ensembl.org).

The phenotypes were treated both as continuous and as binary data (based on a case-control model). In the case of the binary analysis, 133 birds with no or minimal colonisation where considered as controls and the remaining 85 birds were considered as cases.

The AIL data was analysed using GWAS. To investigate any population stratification present in the population, a genomic relationship matrix was generated from all individuals. This genomic relationship matrix was converted to a distance matrix that was used to carry out classical MSA. All analyses of population stratification were performed using GenABEL software [75]. The same software and the mixed model used in the back-cross was also used for the AIL analyses. In addition to sex and experiment, population cluster (1–5) was fitted as a fixed effect to account for population structure. A
Bonferroni correction for multiple testing was applied. After Bonferroni correction significance thresholds were $P \leq 1.75 \times 10^{-7}$ and $P \leq 3.50 \times 10^{-6}$ for genome-wide ($P \leq 0.05$) and suggestive levels, corresponding to $-\log_{10}(P)$ of 6.75 and 5.45, respectively. In addition, a search for SNPs significant at the chromosome-wide level was performed.

**SNP validation**

Individual SNP markers that were significant at the genome- and chromosome-wide level in GWAS were further verified in an association analysis as described for the back-cross. This model also yielded estimates of the magnitude of the significant SNPs effects as described previously by [76].

Finally, the extent of LD among the significant SNP markers was calculated and LD blocks were built as described for the back-cross.

**Further analyses pertaining to both experiments**

**Annotation of the genes in the QTL candidate regions**

The Biomart data mining tool within the Ensembl database (http://www.ensembl.org/biomart/martview/) and the Gal-gal4 assembly were used to locate genes in the candidate regions for *Campylobacter* colonisation resistance identified by the QTL mapping analysis and GWAS for the back-cross and AIL populations. In the case of GWAS, the limits of genomic candidate regions were determined by the LD block structure and the LD present among the significant SNP markers and the neighbouring markers.

The Variant Effect Predictor tool within the Ensembl database (http://www.ensembl.org/Tools/VEP) and the Gal-gal4 assembly were used to determine the location of the significant SNPs identified in the GWAS analyses on the reference genome. The effect of SNPs located in exonic regions on the protein sequence was determined with a SIFT prediction [77] of the protein sequence changes.

**Re-sequencing data analysis**

The two parental lines (6i and N) were fully re-sequenced to 15–20 fold coverage, using pools of 10 individuals per line. Sequencing was performed on Illumina GAIIx platform using a paired end protocol [74]. Re-sequencing data of the candidate regions for *Campylobacter* colonisation resistance, derived from both the back-cross and AIL analysis results, was extracted. More specifically, the extracted sequence included the gene in question and 1 kb upstream and 1 kb downstream of the gene. The SNVs between the two parental lines and the reference genome in these regions were detected using the Mpileup tool for SNP calling (SAMtools v0.1.7). Detected SNVs were annotated using the VEP tool within the Ensembl database (http://www.ensembl.org/Tools/VEP). Information for all SNVs present in the regions of interest was collated. SNV that were in common in the two parental lines but different from the reference genome were filtered out. We separated SNVs into categories based on their relationship to genes. These categories were exonic, intronic, and 1000 bp upstream and downstream. Within the exonic category, there were additional subcategories of UTR and CDS. For SNV’s occurring in CDS regions we also predicted SNV effect as being synonymous, missense, nonsense (stop gain), or splicing. SIFT predictions of the effects of CDS SNVs (tolerated or deleterious) were also acquired through VEP. While mutations in transcribed regions can lead to altered transcriptional products, mutations in regulatory regions can affect transcription rates which ultimately alters phenotype. However, due to the lack of regulatory element annotation in chickens, we developed our own pipeline for predicting TATA boxes and CpG islands. TATA boxes are short DNA regions which act as Transcription Factor Binding sites. CGIs are interspersed DNA sequences that deviate significantly from the average genomic pattern by being GC-rich, and predominantly non-methylated. TATA boxes were predicted by looking for TATA box motifs identified in humans (Computational modeling of oligonucleotide positional densities for human promoter prediction) within the region 20–40 bp upstream of each gene. CpG islands were predicted by using 100 bp windows and scanning across the 1000 bp upstream region with 1 bp intervals to find >200 bp regions with >50 % GC content and an Observed/Expected ratio greater than 0.6 (CpG Islands in Vertebrate Genomes, http://www.bioinformatics.org/sms2/cpg_islands.html). By intersecting our SNV data with our predicted TATA boxes and CpG islands we were able to analyse putative regulatory region mutations.

All the genes, including micro RNAs, were ranked on the rate of non-synonymous coding SNV (dN/L), rate of non-synonymous to synonymous SNV (dN/dS), rate of exonic SNV, rate of intronic SNV.

**Pathway analysis**

The gene lists in the QTL candidate regions for *Campylobacter* colonisation resistance were analysed using the IPA programme (www.ingenuity.com) in order to identify canonical pathways and gene networks constructed by the products of the genes located there. IPA constructs many possible upstream regulators, pathways and networks serving as hypotheses for the biological mechanism underlying the data based on a large-scale causal network derived from the Ingenuity Knowledge Base. Then, IPA infers the most suitable pathways and networks based on their statistical significance, thus a threshold above which the pathways are significant is derived.
Gene expression analysis

Genes whose expression was studied included CXCL1 and CXCL2. Total RNA from a caecal tonsil was isolated from four replicate control birds and eight replicate infected birds per line (6 and N) using the RNaseasy Mini Kit (Qiagen West Sussex, United Kingdom) following the manufacturer’s instructions. For each gene, mRNA levels were quantified using TaqMan quantitative RT-PCR. Primers and probes for the 28S rRNA, CXCL1 and CXCL2 genes were as described previously [78, 79]. TaqMan assays were performed as described by Sutton et al. [80]. Data was expressed as cycle threshold (Ct) value, which was normalised using the formula: \( \text{Ct} + (N - t - C_t)\) where \( N \) is the mean Ct value for 28S RNA in all samples, \( t \) is the mean value for 28S RNA in the sample and the \( S \) and \( S' \) are the slopes of regression of the standard plots for the specific cytokine or chemokine mRNA and the 28S RNA, respectively. Expression data was calculated as fold changes compared with the mean of the control mock-infected samples. Results are expressed as the mean of the fold changes between replicate samples and error bars represent the standard error of this mean. Differences between control and infected samples were examined with an analysis of variance.

Ethics

All animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986, with the approval of the Ethical Review Committee of The Pirbright Institute (under project licence PPL 30/2462) and the Animal Welfare and Ethical Review Body of The Roslin Institute (under PPL 60/4420).

Availability of supporting data

The SNP data used in this study is available in NCBI dbSNP at the following web page: http://www.ncbi.nlm.nih.gov/SNP/snp_viewBatch.cgi?sbid=1062063. The same data is also available at the National Avian Research Facility website through the link: http://www.narf.ac.uk/VariationDatabase.

Additional files

- **Additional file 1: Table S1** Levels of C. jejuni colonisation in the caeca of line 6, line N, back-cross and advanced intercross line birds. The measurements took place one, two, three, four and five days after challenge at 3 weeks of age. Data are presented as mean log_{10} (CFU/g) ± standard deviation of the mean. (XLSX 12 kb)
- **Additional file 2: Figure S1** Manhattan plot and Q-Q plot displaying the GWAS results from the back-cross experiment. Genomic location is plotted against -log_{10}(P) in the Manhattan plot (above). Suggestive genome-wide threshold is shown as a horizontal line. Q-Q plot (below) of observed \( P \)-values against the expected \( P \)-values for Campylobacter gut colonisation (log-transformed number of C. jejuni per gram of caecal contents). (PPTX 1422 kb)

**Abbreviations**

AIL: advanced intercross line; B:BTNL2: Butyrophilin subfamily 2 member A2; BFV1/1: MHIC class I alpha chain II; BG1: MHIC class IV antigen (B-G); BLEC-2: B-cell CLL/Lymphoma 2; C: jejuni: Campylobacter jejuni; C16orf96: chromosome 16 open reading frame 96; C4: complement 4 precursor; CCDC108: coiled-coil domain containing 108; CDH11: cadherin 11 precursor gene; CDH3: T-cadherin; CDH5: cadherin 5-type 2; CDH8: cadherin 8-type 2; C: jejuni: Campylobacter jejuni; Ct: cycle threshold; CV: coefficient of variation; dN/dS: rate of non-synonymous to synonymous SNV; dN: number of non-synonymous SNV divided by the length of the coding region of the gene; EPHA: ephrin receptor A5; EPHA: ephrin receptor A5; GLBNL: galactosidase, beta 1-like; GWAS: genome-wide association study; IBV: infectious bronchitis virus; IFH1: interferon-induced helicase C domain-containing protein 1; IL4I1: interleukin 4 induced 1; LD: linkage
dissequilibrium; LY75: lymphocyte antigen 75 precursor; MAF: minor allele frequency; MDV: Marek's disease virus; MHC: major histocompatibility complex; MSA: multidimensional scaling analysis; NAT75: N-acetyltransferase 15; NARMP1 (SLC11A1): natural resistance-associated macrophage protein 1; OBSL1: Obozoorin-like 1; PP1RN: protein tyrosine phosphatase, receptor type, N; QTL: quantitative trait loci; RACGAP1: Rac GTPase-activating protein 1; SLC38A1: solute carrier family 38 member 11; SNP: single nucleotide polymorphism; SNV: single nucleotide variant; SPEG: SPEG complex locus; SPF: specified pathogen-free; TAP2: antigen peptide transporter 2; TRAP: Tumour necrosis factor receptor-associated protein 1; TRIM: TRAP-related; TTRM27: Tryptophan mott-containing 27; T7CC21B: Tetratricopeptide repeat domain 21B; UTR: untranslated region; ZNF142: Zinc finger protein 142.

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

Authors’ contributions
PK, MS, MJ, NS conceived and designed the backcross study and secured substantial funding; PK, MS, MF, conceived and designed the AIL study and secured substantial funding; MS and MJ developed the infection models used in this study. JH, NS, MF and PDM performed the experimental challenge, the phenotyping of the back-cross experiment and the cytokine expression work; JH, NS, MF created the SNP set and genotyped the backcross DNA; MF, PMH, NS and AP collated, edited and performed the genetic analysis of the backcross data; AP, GB, OM collated, edited and performed the genetic analysis of the AIL data; RK annotated the sequence data, AP, RK and GB analysed the re-sequencing data; AP and JS performed the pathway analyses; JS analysed the expression of candidate genes in other viral infections; PK, DH and NS helped with the interpretation of the results. AP wrote the manuscript. All other co-authors provided manuscript editing and feedback. All authors read and approved the final manuscript.

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Page 16 of 18
Psifidi et al. BMC Genomics (2016) 17:293
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