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Specificity of resistance to dengue virus isolates is associated with genotypes of the mosquito antiviral gene Dicer-2

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In contrast to the prevailing view that invertebrate immunity relies on broad-spectrum recognition and effector mechanisms, intrinsic genetic compatibility between invertebrate hosts and their pathogens is often highly specific in nature. Solving this puzzle requires a better understanding of the molecular basis underlying observed patterns of invertebrate host–pathogen genetic specificity, broadly referred to as genotype-by-genotype interactions. Here, we identify an invertebrate immune gene in which natural polymorphism is associated with isolate-specific resistance to an RNA virus. Dicer-2 (dcr2) encodes a key protein upstream of the RNA interference (RNAi) pathway, a major antiviral component of innate immunity in invertebrates. We surveyed allelic polymorphism at the dcr2 locus in a wild-type outbred population and in three derived isofemale families of the mosquito Aedes aegypti that were experimentally exposed to several, genetically distinct isolates of dengue virus. We found that dcr2 genotype was associated with resistance to dengue virus in a virus isolate-specific manner. By contrast, no such association was found for genotypes at two control loci flanking dcr2, making it likely that dcr2 contains the yet-unidentified causal polymorphism(s). This result supports the idea that host–pathogen compatibility in this system depends, in part, on a genotype-by-genotype interaction between dcr2 and the viral genome, and points to the RNAi pathway as a potentially important determinant of intrinsic insect-virus genetic specificity.

1. Introduction

Intrinsic compatibility between invertebrate hosts and their pathogens often depends on the specific pairing of genotypes [1,2]. This genetic specificity, referred to as genotype-by-genotype interactions, has been documented in a wide variety of invertebrate host–pathogen systems [3–9] and can reach extreme levels [10]. This observation is in contrast with the conventional view that invertebrate immune systems only respond differently to broad classes of pathogens, for example, Gram-positive and Gram-negative bacteria [11]. To resolve this apparent paradox, a better understanding of the molecular basis underlying observed genotype-by-genotype interactions between invertebrates hosts and pathogens is required [12,13]. Molecular determinants of host–pathogen specificity have been well described in plant-pathogen systems [14,15] and in the case of compatibility patterns between genetically diverse pathogens and major histocompatibility complex (MHC) variants of vertebrates [16,17]. With a few exceptions [18–21], the genetic basis of invertebrate host–pathogen compatibility is poorly understood. Identifying the mechanisms underlying host–pathogen genetic specificity has important implications for a broad spectrum of evolutionary, epidemiological and medical phenomena [1,13].
In the present study, we examined the role of an invertebrate immune gene in strain-specific resistance to an RNA virus. Dicer-2 (dcr2) encodes a ribonuclease acting upstream of the RNA interference (RNAi) pathway [22], a major antiviral component of innate immunity in invertebrates [23–25]. Building on a previous study that revealed strong genetic specificity in the interaction between *Aedes aegypti* mosquitoes and dengue viruses (DENV) in a natural situation in Thailand [5], we sought to determine whether genetic polymorphism at the dcr2 locus could explain, in part, the observed compatibility pattern. DENV (serotypes 1–4) are mosquito-borne RNA viruses of the genus *Flavivirus* that cause a spectrum of clinical manifestations ranging from mild febrile illness to life-threatening haemorrhagic fever, and are the most important insect-borne viral infection of humans [26]. *Aedes aegypti* is the primary mosquito vector of DENV worldwide [26].

We genotyped dcr2 and two flanking loci (AAEL006790 and AAEL006800; figure 1a) in a wild-type *Ae. aegypti* population from Ratchaburi, Thailand and in three independent isofemale families (i.e. the progeny of individual females) derived from this population. AAEL006800 is a gene encoding a sodium/chloride-dependent transporter located approximately 11.5 kb downstream of dcr2, and AAEL006790 is a hypothetical protein-coding gene located approximately 92 kb upstream of dcr2 (figure 1a). They were chosen in the close vicinity of dcr2 as ‘negative controls’ to verify the locus-specificity of genotype–phenotype associations tested at the dcr2 locus. AAEL006800 is the closest gene downstream of dcr2, whereas two other short genes (AAEL006801 and AAEL006808) are located between dcr2 and AAEL006790. The outbred population and the isofemale families represent two complementary approaches to assess genotype–phenotype associations. The outbred population provides a representative sample of the natural genetic diversity at a locus, but has reduced power to detect genotype–phenotype associations at this locus because the genetic background, including other genes underlying the phenotype, is diverse. In other words, background variation in the outbred population results in a stringent, although less sensitive, test of genotype–phenotype associations. Conversely, isofemale families display restricted overall genetic diversity (a maximum of four parental alleles per locus), and increased power to detect genotype–phenotype associations because siblings share a relatively homogenous genetic background. We measured the probability of viral infection of midgut epithelial cells and subsequent dissemination to secondary tissues, which are two essential steps for DENV transmission by *Ae. aegypti* bite. Both events are prerequisites for virus transmission and have been used to define a ‘midgut infection barrier’ and a ‘midgut escape barrier’ underlying *Ae. aegypti* resistance to DENV [27].

**2. Material and methods**

**(a) Mosquitoes and viruses**

A laboratory *Ae. aegypti* population was established with a large number (more than 1500) of immatures (larvae and pupae) collected during 2007 in Ratchaburi, Thailand [5]. Mosquitoes were maintained as a large, randomly mating population (more than 1800 adults per generation) under standard conditions for two generations to minimize the influence of parental effects that can be confounded with genetic effects [28]. We also derived three independent *Ae. aegypti* isofemale families (denoted as A, B and C) generated as the progeny of three inseminated females randomly chosen from the outbred population. Families A, B and C were represented by 125, 112 and 96 individual females, respectively. We examined statistical associations between genetic polymorphism and infection phenotype both in the parental, outbred population and within the isofemale families. We exposed mosquitoes to an artificial DENV infectious blood meal as described previously [5]. The parental population was exposed to two DENV-1 isolates collected in Ratchaburi, Thailand in 2007 (RTB-138 and RTB-196) represented by 20 and 40 phenotyped *Ae. aegypti* females, respectively. The three families were exposed to three other DENV-1 isolates (BKK, KPP and RTB) collected during 2007 in Thailand [5], resulting in 28–53 (mean 37) phenotyped females per family-isolate pair. Prior to their use in experimental infections, all five isolates were passaged five times in *Aedes albopictus* (C6/36) cells, with the exception of RTB-196 that was passaged two times in *Toxorhynchites splendens* mosquitoes and three times in C6/36 cells.

Two conventional indices of mosquito resistance to DENV were measured: (i) the proportion of mosquitoes that became infected and (ii) the proportion of infected mosquitoes (excluding

![Figure 1. Allelic polymorphism in the dcr2 genomic region of *Aedes aegypti*.](image-url)
uninfected ones) that developed a disseminated infection (virus was detected in their head or their legs) 14 days after the infectious blood meal at 28°C [5]. These genotypes are defined as the Ae. aegypti ‘midgut infection barrier’ and ‘midgut escape barrier’ to DENV transmission that were previously described [27]. The complete genome sequences of BKK, KPP and RTB virus isolates (GenBank accession nos HM469966, HM469967 and HM469968, respectively), showed 2.4 per cent overall nucleotide divergence and 1.8 per cent mean pairwise nucleotide divergence. The complete genome sequences of isolates RTB-138 and RTB-196 are not available yet, but the sequence of more than 96 per cent of their genomes showed similar levels of nucleotide divergence. Because the infectious blood meal offered to mosquitoes was standardized (including the infectious dose), isolate was used as a proxy for virus genetic identity.

Phylogenetic relationships between the five DENV-1 isolates in the study and a representative set of DENV-1 isolates previously collected in southeast Asia were examined by focusing on the coding part of the viral genome (see alignment in the electronic supplementary material, file S1). Absence of recombination events was verified with the RDPh software [29]. Phylogenetic relationships were inferred with MEGA5 [30] using the maximum-likelihood method and the general reversible model (GRT + I + I) based on a Gamma discrete distribution and a 3.63 per cent proportion of invariant sites. The bootstrap consensus tree was based on 500 replicates and the few nucleotide positions containing gaps were ignored.

(b) Mosquito genotyping

The dcr2 gene (ID AAEL006794 in VectorBase: http://aaegypti.vectorbase.org/) was genotyped by amplifying a 572-bp region located at positions 1559–2106 in the transcript sequence (AAEL006794-RA) consisting of (i) 205 bp in exon 7, transcript positions 1599–1803, located in a putative helicase domain; (ii) a 64-bp intron; and (iii) 303 bp in exon 8, transcript positions 1804–2106, in a putative dsRNA binding domain. Specific forward and reverse PCR primers were as follows: FOR 5'-CGTGTAGAGGAGGGATTG, REV 5'-CAACTTGATATTGC.

All sequencing chromatograms were aligned in Vectorbase.org (b) Mosquito genotyping

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Seven biallelic single nucleotide polymorphisms (SNPs) were identified (1806C>T, 1839C>T, 1840C>A, 1851C>T, 1884G>A, 2004G>C and 2029A>G), where numbers refer to the transcript position and letters indicate the most and least frequent alleles, respectively. All are synonymous substitutions because SNPs are most often biallelic and, therefore, might not faithfully represent the true allelic diversity of the locus. The number of alleles identified is contingent on the number of SNPs considered per locus, which in turn depends on the size of the PCR product. We arbitrarily chose a number of SNPs that provided sufficient resolution to discriminate at least four alleles (i.e. the maximum number of alleles expected to be found in the isofemale families).

(c) Population genetics

The occurrence of linkage disequilibrium (LD) was tested for each pair of loci in the outbred population using exact probability tests for genotypic disequilibrium (batches = 5000; iterations = 20000; dememorization = 1000) implemented in GENEPOP v. 4.0 [33]. Recombination parameters were estimated in PHASE v. 2.1.1 [34] using the general model for recombination rates of multi-allelic loci other than microsatellites (i.e. that does not assume a stepwise mutation model). All default priors were unchanged with the exception of a prior background recombination parameter of 2 × 10⁻⁵. Different prior values (range 4 × 10⁻⁵–8 × 10⁻⁵) for the background recombination parameter did not qualitatively change the results. The final run was iterated 10 times (all loci). Results shown are those with the best average goodness-of-fit from 10 independent runs of the algorithm. The possibility that laboratory colonization resulted in a demographic bottleneck was examined in the outbred population using two complementary analyses. First, because allele number decreases faster than heterozygosity when populations shrink in size, a signature of bottlenecks observed in subsequent generations is Hs > Hneq where Hs is the expected heterozygosity in the population of interest under Hardy–Weinberg equilibrium and Hneq is the expected heterozygosity in a population with the same sample size and allele number at mutation-drift equilibrium [35]. This analysis was performed using BOTTLENECK v. 1.2 [36]. Second, we calculated Tajima’s D and Fu and Li’s D* statistics for each of the three genes in DnaSP v. 5.0 [32]. Negative values of D and D* indicate an excess of rare alleles relative to expectation under demographical stability and quasi-neutral evolution, and are interpreted as a recent population bottleneck (or a selective sweep in a demographically stable population).

(d) Genotype–phenotype associations

For the outbred population, only the two most common genotypes of each locus were included in the genotype–phenotype association analyses because the sample sizes for other genotypes were too small for a meaningful analysis. The proportion of infected mosquitoes and the proportion of infected mosquitoes that developed a disseminated infection (excluding uninfected ones) were analysed using a nominal logistic regression that included the effects of genotype, isolate, infectious dose and their interactions up to the second order. The isolate × infectious dose interaction was omitted from the model because one of the two isolates had only been used at one virus concentration and, therefore, this interaction could not be tested. For the isofemale families, the analysis was conducted in two steps. First, the proportion of infected females and the proportion of infected females with a disseminated infection were analysed with a nominal logistic regression that included the effects of family, isolate, genotype and their interactions up to the second order. Genotype was nested within family because each family contained a different set of genotypes. Second, because the main effect of the family proved insignificant in the initial analysis, the same analysis was performed without including the family, so that all genotypes
were pooled regardless of family. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed with *JMP* v. 10.0 (SAS Institute Inc., NC, USA).

Full datasets, including phenotypes, genotypes and individual SNP calls are provided for both the outbred population (see the electronic supplementary material, file S2) and the isofemale families (see the electronic supplementary material, file S3).

### 3. Results

Phylogenetic analysis of the coding region of viral genomes detected no major recombination event among the five DENV-1 isolates used in the present study and other viruses that recently circulated in Thailand. The five DENV-1 isolates of the study belong to two sister groups nested within a large phylogenetic lineage referred to as genotype I (figure 2). All five isolates are closely related to viruses that circulated during 2001 in Bangkok and Kamphaeng Phet (see BKK01 and KPP01 in figure 2). Four of the five isolates cluster together in one sister group, whereas the KPP isolate diverged slightly earlier and belongs to the other sister group. Pairwise differences between isolates were nucleotide substitutions scattered throughout the viral genome (see the electronic supplementary material, file S1).

Single nucleotide polymorphisms (SNPs) surveyed at the *dcr2* locus in a sample of 100 individuals of the outbred *Ae. aegypti* population defined 17 genotypes resulting from 10 different *dcr2* alleles (provisionally named 01 to 10) with frequencies ranging from 0.5 to 61.5 per cent (figure 1b). Flanking genes displayed a similar pattern of polymorphism with 11–12 genotypes based on six to eight alleles per locus (figure 1b). Consistent with their close physical proximity, genotypic LD was significant between each pair of loci (*dcr2*-AAEL006790: $p = 0.0017$; *dcr2*-AAEL006800: $p = 0.0023$; AAEL006790-AAEL006800: $p = 0.0433$). Fifteen of the 42 possible pairs of individual SNPs resulted in

![Figure 2. Phylogenetic relationships among DENV-1 isolates used in this study. Background genomic sequences retrieved from GenBank are labelled with their accession number followed by an indication of their geographical origin (Camb, Th, BKK and KPP stand for Cambodia, Thailand, Bangkok and Kamphaeng Phet, respectively), and by two digits referring to the sampling year. Major clades are labelled according to previously described DENV-1 ‘genotypes’. The five DENV-1 isolates used in this study are indicated by green terminal branches. They were isolated in 2007 from three different Thai localities: Bangkok (BKK), Kamphaeng Phet (KPP) and Ratchaburi (RTB, RTB-138, RTB-196). The inset in the upper left corner shows the position of the DENV-2 isolate that was used to root the tree. Bootstrap support values are shown next to the relevant nodes.](rsb.royalsocietypublishing.org)
significant LD between dcr2 and AAEL006790, whereas 8 of the 42 possible pairs of SNPs showed significant LD between dcr2 and AAEL006800. The median value of recombination rate estimated for the dcr2 genomic region was 4 × 10^{-7} per generation (95% CI: 1.0 × 10^{-8}–3.7 × 10^{-8}). Assuming an effective population size between 500 and 1000 individuals, this corresponds to 0.1–0.2% recombination per Mb, in agreement with a previous genome-wide estimate of 0.15 per cent recombination per Mb [37,38]. There was no indication of a population bottleneck that may have occurred during laboratory colonization of the population. Heterozygosity calculated from the allele frequencies (H_e) at the three loci did not deviate significantly from expected heterozygosity at mutation-drift equilibrium (H_eq), with a tendency for a heterozygosity deficit instead of the expected heterozygosity excess following a population bottleneck (dcr2), with a median value of recombination of 4.57, d.f. = 1, p = 0.02325). Because the interaction effect resulted in a similar pattern for infection and dissemination, for the sake of simplicity, the cumulative probability of infection + dissemination is shown in figure 3. Although the infection + dissemination probability of both isolates was similar (approx. 50%) for mosquitoes with genotype 01–01, it was strikingly different for mosquitoes with genotype 01–10 (figure 3). Among mosquitoes with genotype 01–10, none had a disseminated infection with isolate RTB-138, whereas almost 90 per cent had a disseminated infection with isolate RTB-196.

Because of the significant LD, distribution overlap between the two most common dcr2 genotypes and the two most common genotypes at the control loci was 76.3 per cent for AAEL006790 and 88.1 per cent for AAEL006800. In contrast to the dcr2 locus, however, no genotype × isolate interaction associated with infection probability was detected at either control locus when considering their two most common genotypes (AAEL006790: LR \chi^2 = 0.26, d.f. = 1, p = 0.6090; AAEL006800: LR \chi^2 = 0.05, d.f. = 1, p = 0.8186) despite an increase in statistical power due to larger sample sizes (n = 61, n = 74 and n = 79 for dcr2, AAEL006790 and AAEL006800, respectively). The genotype × isolate
Figure 4. Isolate- and locus-specific association between viral dissemination and dcr2 genotype in the mosquito families. Interaction plot showing the percentage of infected mosquitoes in the three isofemale families that developed a disseminated viral infection from three different DENV isolates (green, BKK; blue, KPP; red, RTB) as a function of the genotype at the dcr2 locus (b) and at the two control loci (a,c). Dotted, vertical bars are 95% CI of the percentages. P-values above the graphs indicate the statistical significance of the genotype × isolate interaction term in a two-way logistic regression accounting for the effects of isolate and genotype. Genotypes from the three families are pooled because the main effect of family was insignificant and thus removed from the analysis. Note that for purposes of clarity, one data point (KPP isolate, genotype 01–03) was omitted in (a) because it consisted of only two individuals.
locus-specific association detected between the dcr2 genotype and the viral dissemination phenotype in the outbred population was also found in the isofemale families exposed to three additional DENV-1 isolates.

4. Discussion

Although the occurrence of specific genetic interactions between invertebrate hosts and their pathogens is ubiquitous in nature, the underlying molecular basis has rarely been defined. In the present study, we document a statistical association consistent with a genotype-by-genotype interaction between the mosquito antiviral gene dcr2 and DENV. The dcr2 locus encodes the ribonuclease Dicer-2 that acts upstream of the RNAi pathway, an important antiviral defence mechanism of plants and invertebrates [39]. Dicer-2 recognizes and cleaves long double-stranded RNA (dsRNA) molecules resulting from secondary structures or replication intermediates. It processes viral dsRNA into 21–23 bp small interfering RNAs (siRNAs), which are incorporated into an RNA-induced silencing complex (RISC), leading to sequence-specific degradation of the target viral RNA [22]. Transient silencing of key genes of the RNAi pathway, including dcr2, results in a significant increase in DENV-2 titres in Ae. aegypti mosquitoes [24]. Constitutive impairment of the RNAi pathway also results in increased dissemination of Sindbis virus from the midgut of Ae. aegypti [40]. In Drosophila, Dicer-2 displays an exceptionally elevated rate of amino acid substitution, suggestive of rapid evolution through natural selection by RNA viruses [41,42]. It is worth noting that the low DENV prevalence in wild Ae. aegypti populations [43] and a relatively modest fitness cost of infection [44] make it unlikely that dcr2 polymorphism is primarily shaped by DENV-mediated selection.

Our analyses were based on the same tests of genotype–phenotype associations at one candidate gene, dcr2 and two control genes flanking the dcr2 locus. Although statistical power was identical or greater for the control genes than for the candidate gene, dcr2 was the only locus for which the interaction between mosquito genotype and virus isolate significantly influenced the outcome of infection. Our results point to the RNAi pathway as a possibly important determinant of intrinsic insect-virus genetic specificity. This is one of the few candidate immune genes underlying strain-specific resistance identified in a natural invertebrate population. This finding highlights the difficulty associated with the genetic mapping of a locus involved in strain-specific resistance. Because the effect of such a locus is most apparent by comparing resistance patterns across several pathogen strains, it will often go undetected by conventional mapping strategies that use a single pathogen strain [45]. Mapping loci involved in specific host–pathogen interactions requires that multiple combinations of host and pathogen genotypes are considered simultaneously [13].

Although alternatives are unlikely, our study design does not allow us to conclusively assign a causal role to the dcr2 locus because the statistical association could result from undetected LD between dcr2 and the causal polymorphism(s). Below, we discuss three potential scenarios. (i) Large chromosomal inversions may link physically distant genetic loci. Existence of inversion polymorphisms has been hypothesized in Ae. aegypti because some genomic regions have a reduced recombination rate [46]. Direct evidence for segregating chromosomal inversions, however, has only been detected in the forest form Ae. aegypti formosus, which is found in West Africa [47]. To the best of our knowledge, segregating chromosomal inversions have not been detected among wild populations of the domestic form Ae. aegypti that we used in our study. If dcr2 is located within a chromosomal inversion, flanking genes would remain valid controls. In the unlikely event that the inversion breakpoint was located precisely at the dcr2 locus, at least one of the two flanking genes would remain a valid control locus. In contrast to this hypothesis, both flanking loci showed similar levels of LD with dcr2 in the outbred population and we failed to detect the genotype–phenotype association found at the dcr2 locus. (ii) A demographic bottleneck during laboratory colonization of the mosquito population may have artificially increased LD. Maintaining a large population size at each generation since the initial field collection, however, should have limited genetic drift during the colonization process. Accordingly, population genetic parameters in the outbred population did not display any signature of a recent demographic bottleneck. (iii) Long-range LD may exist between dcr2 and a physically unlinked locus due to epistatic selection [48]. In this case, the dcr2 locus would be in stronger LD with one or more distant gene(s) than with physically close loci because of functional relationships between the genes. Even under this scenario, however, our conclusion that polymorphism at the dcr2 locus influences the phenotype would remain valid.

We do not expect that the dcr2 locus is solely responsible for the variation in Ae. aegypti resistance to DENV, which is known to be a polygenic trait [27]. In the present study, a variance component analysis estimated that the specific dcr2 genotype × virus isolate interaction explained 17.8 per cent of the total variance in the probability of viral dissemination among the isofemale families. The existence of other genetic factors influencing mosquito resistance to DENV may help to explain why in the isofemale families dcr2 genotype was only associated with isolate-specific probability of viral dissemination from the midgut to the rest of the mosquito body, but not with midgut infection probability. In the outbred population, dcr2 genotype influenced both infection of midgut epithelial cells and subsequent dissemination to secondary tissues. We speculate that the isofemale families may have captured a subset of polymorphisms at other genetic loci involved in resistance to DENV that were not fully representative of the outbred population.

The underlying mechanism of virus isolate-specific resistance of different dcr2 genotypes remains to be characterized. We hypothesize that non-synonymous polymorphisms within dcr2 may result in differential dsRNA binding affinities of the Dicer-2 protein for particular dsRNA sequences and thus differential recognition of particular viral strains, leading to variation in the efficiency of RNAi-mediated antiviral activity. This hypothesis is supported by results of an in vitro study that detected significant variation among different DENV strains in their sensitivity to Dicer-2 knock-down [49]. Alternatively, different viral RNAI suppressors may differentially affect the activity of Dicer-2 allelic variants [23,50,51]. Future studies will dissect the mechanistic basis of Dicer-2 implication in insect-virus genetic specificity.
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