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HOST MICROBE INTERACTIONS

Honey Bee Habitat Sharing Enhances Gene Flow of the Parasite *Nosema ceranae*

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Abstract
Host-parasite co-evolution is a process of reciprocal, adaptive genetic change. In natural conditions, parasites can shift to other host species, given both host and parasite genotypes allow this. Even though host-parasite co-evolution has been extensively studied both theoretically and empirically, few studies have focused on parasite gene flow between native and novel hosts. *Nosema ceranae* is a native parasite of the Asian honey bee *Apis cerana*, which infects epithelial cells of mid-guts. This parasite successfully switched to the European honey bee *Apis mellifera*, where high virulence has been reported. In this study, we used the parasite *N. ceranae* and both honey bee species as model organisms to study the impacts of two-host habitat sharing on parasite diversity and virulence. SNVs (Single Nucleotide Variants) were identified from parasites isolated from native and novel hosts from sympatric populations, as well as novel hosts from a parapatric population. Parasites isolated from native hosts showed the highest levels of polymorphism. By comparing the parasites isolated from novel hosts between sympatric and parapatric populations, habitat sharing with the native host significantly enhanced parasite diversity, suggesting there is continuing gene flow of parasites between the two host species in sympatric populations.

Keywords Honey bee · Microsporidia · Genetic diversity · Selection

Introduction

Host-parasite co-evolution is a process of reciprocal selection and adaptive genetic change. The genetic mechanisms underlying co-evolution provide a central paradigm in evolutionary biology [23]. During co-evolution, parasites may also explore external resources by shifting to other host species. Successful host shifting is affected by phylogenetic distance, with closely related host species most likely to share parasites [11]. After establishing a sustainable relationship with the novel host, a new round of genetic oscillation starts. In natural conditions, the parasites may switch back to the native host, given both species share a habitat.

Nosema ceranae is a native microsporidian parasite of the honey bee *Apis cerana* that infects epithelial cells of honey bee mid-guts [15]. The spores germinate in the mid-gut and inject infective sporoplasm into host cells with an extruded polar filament. The ensuing intracellular reproduction cycle lasts ~ 4 days. Proliferation in cells starts from meronts, leading to sporonts and finally the formation of mature spores [25]. The infected cell eventually bursts, releasing large numbers of spores. Following a successful switch to another honey bee species, *Apis mellifera, N. ceranae* has been implicated in colony losses for this widespread species [3, 9, 26]. Since *A. mellifera* and *A. cerana* coexist in Asia and Australia, the parasite *N. ceranae* has the opportunity to infect and transmit between these two honey bee species. We hypothesize that habitat sharing by host species facilitates the escape of parasites. When escape events are frequent, this can lead to high genetic diversity in the sympatric novel host, *A. mellifera*. In order to test the above hypothesis, we first validated the gene flow of these parasites between the two sympatric honey bee species. Then, the impact of host habitat sharing on parasite diversity was quantified by comparing parasite genetic diversity from novel hosts of sympatric and parapatric populations. Thirdly, immune responses of the native host towards parasites isolated from

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novel and native hosts were quantified to test for any virulence selection.

Methods

Host and parasite populations

In China, the two honey bee species *A. mellifera* (Western honey bee) and *A. cerana* (Eastern honey bee) share habitats and are defined as sympatric populations. In Europe, only *A. mellifera* is available since *A. cerana* has not been introduced. So, *A. mellifera* honey bees in China and Europe are defined as parapatric populations. Overall, three sources of parasites were analyzed, including parasites isolated from native and novel hosts in sympatric populations and parasites isolated from the novel host in a parapatric population.

Parasite isolation and sequencing

Approximately 40 honey bee foragers were collected from each of five *A. mellifera* and *A. cerana* colonies from sympatric populations in China. First, mid-guts of the collected honey bees from each colony were dissected, pooled and homogenized to isolate *N. ceranae* spores following a standard protocol [16]. Then, the isolated spores were further purified using Percoll gradient centrifugation [8]. Spores were counted using a haemocytometer and confirmed as pure *N. ceranae* by species-specific PCR [16]. After that, the spores from 5 *A. mellifera* and 5 *A. cerana* colonies were pooled separately, leading to two pools of parasite spores. As the genetic diversity of *N. ceranae* is higher within a colony than among colonies, 5 colonies could represent the genetic diversity of a local region [19–21]. Genomic DNA derived from these spore pools was extracted using CTAB (cetyl trimethylammonium bromide) [8]. One sequencing library was constructed from each of the two DNA pools for whole genomic sequencing. Finally, these two libraries were prepared and sequenced using the BGISEQ platform (Beijing Genomics Institute Sequencing).

SNVs analysis and statistics

For each of the two libraries, over 3.5 million paired reads (150 nucleotides per read) were successfully aligned to the *N. ceranae* genome assembly version GCA_004919615.1 [29] using Burrows-Wheeler Aligner (BWA) with default parameters [35]. In addition, sequencing reads of *N. ceranae* isolated from *A. mellifera* (Spain PA08 1199) were downloaded from the NCBI SRA archives for comparative analyses [41, 42]. The spores were isolated from 40 *A. mellifera* worker honey bees. Over 2.8 million reads (100 nucleotides per read, paired end, Illumina Hiseq) were successfully aligned to the *N. ceranae* genome, using BWA with default parameters. All three data sets provide over 30-fold coverage of the genome and neither of the sequencing platform has reported systematic bias. Therefore, the reads enable comparable and accurate identification of SNVs among the three data sets. The SNVs were identified and annotated using the Picard-GATK-SNPEFF pipeline [53]. Only the SNVs in coding regions were further analyzed (synonymous and non-synonymous SNVs). The numbers of SNVs per gene were compared between parasite sources using the paired t-test with R. Allele frequency differences among populations were analyzed using ANOVA. The ratio of $\pi_{nonsynonymous}/\pi_{synonymous}$ (π_d/π_s) was calculated using SNPGenie [40]. *Watterson's* θ and corrected *Tajima's D* were calculated using PoPoolation [32]. The genome diversity π and the fixation index F_{st} were calculated using PoPoolation2 [31]. All raw reads have been deposited in NCBI under BioProject PRJNA602377 with accession # SAMN13893430 and SAMN19893431. Detailed bioinformatic methods are provided in supplementary materials file S1.

Nosema ceranae spore inoculation

Sealed brood frames of *A. cerana* were kept in an incubator (34 ± 1 °C, 60% relative humidity). Sixty-eight freshly emerged honey bee workers were individually fed with 2 µl sucrose solution containing 10^5 *N. ceranae* spores isolated from the native host in China. An additional 68 *A. cerana* workers were fed with 2 µl sucrose solution containing 10^5 *N. ceranae* spores isolated from the novel host (*A. mellifera*) in China. Furthermore, 68 *A. cerana* workers were fed with sugar water as an uninfected control. During the experiment, cohorts were divided into two cups containing 34 bees each and maintained on 50% sucrose solution ad libitum in the incubator.

Quantitative real time PCR

Four bees were collected from each rearing cup at 24 h interval from 1 to 5 days post infection (dpi). Total RNA was extracted from mid-gut tissue with Trizol from each individual bee. Equal amounts of RNA from two bees of the same cup were pooled for cDNA synthesis and qPCR with 4 biological replicates. Thirty-nine previously reported honey bee immune genes covering all four immune pathways together with two reference genes (*β-Actin*, *GAPDH*) were quantified for all samples from 1 to 5 dpi [12] (Table S1). Immune genes and reference genes for each sample were run in the same 96 well plate to avoid plate variance. Purified water was used as a negative control to rule out contamination. Each gene per sample was run in duplicate. The detailed qPCR procedure is provided in the supplementary material S1.

RT-qPCR data analysis

C_t values and PCR amplification efficiencies were calculated for individual reactions with the qpcR package based on the strength of fluorescence on each qPCR cycle [49]. The mean amplification efficiency of each gene over all samples was used for subsequent calculations. The mean of both reference genes was used to calculate relative expression levels for the target genes [28]. The relative expression differences between groups were analyzed using t-tests, corrected for multiple measurements using false discovery rates, with R [44].

Results

Genetic analysis of the parasite from sympatric populations

Overall, 112,809 and 113,408 SNVs were identified in the parasite isolated from native and novel hosts in sympatric populations respectively (Fig. 1). The density of overall SNVs (number of SNVs per 10 Kb) did not significantly deviate between the two parasite isolates along contigs ($P > 0.05$, Paired t-test). The number of synonymous and non-synonymous SNVs was not significantly different between the two isolates ($P > 0.05$, Pearson's Chi-squared test). The ratio of π_a / π_s was < 1 in both isolates (Table 1). Parasite genome diversity (π) and *Watterson's* θ were higher in the native host (0.0098 ± 0.0001 for π and 0.0087 ± 0.0001 for θ) than the novel host (0.0089 ± 0.0001 for π and 0.0074 ± 0.0001 for θ). The *Tajima's D* was positive in both isolates, with higher values in the novel sympatric host (1.447 ± 0.018) compared with the native host (0.994 ± 0.014) (Table 1). The fixation index F_{st} was small (0.0134 ± 0.0003) between the two sympatric populations (Table 2).

Genetic analysis of the parasite from parapatric populations

We further compared parasites isolated from the novel host species *A. mellifera* in China with those from a parapatric population in Spain. In total, 53,974 SNVs were identified in parasites isolated from *A. mellifera* in Spain, which was significantly less than 113,408 SNVs identified from parasites isolated from *A. mellifera* in China ($P < 0.001$, Paired t-test). The proportion of non-synonymous SNVs was significantly higher in the parasite in China compared with those in Spain ($P < 0.001$, Pearson's Chi-squared test, Table S1). The ratio of π_a / π_s was < 1 in both isolates (Table 1). The parasite genome diversity (π) and *Watterson's* θ were higher in novel host China (0.0089 ± 0.0001 for π and 0.0074 ± 0.0001 for θ) than novel host in Spain (0.0028 ± 0.0001 for π and 0.0025 ± 0.0001 for θ). The *Tajima's D* was again positive in both isolates, where the value is higher in the novel host China (1.447 ± 0.018) compared with the novel host Spain (0.350 ± 0.022) (Table 1).

Allele frequency analysis among three populations

Overall, 6989 synonymous and 5279 non-synonymous SNVs were shared among the three *N. ceranae* populations (Fig. 1). All the SNVs were biallelic. A significant difference in the allele frequency was not found from the non-synonymous SNVs among the three parasite populations. However, the allelic frequency of synonymous SNVs was significantly different among the three parasite populations ($P < 0.01$, ANOVA) (File S2). The parasite isolated from the novel host in Spain showed a significantly higher number of

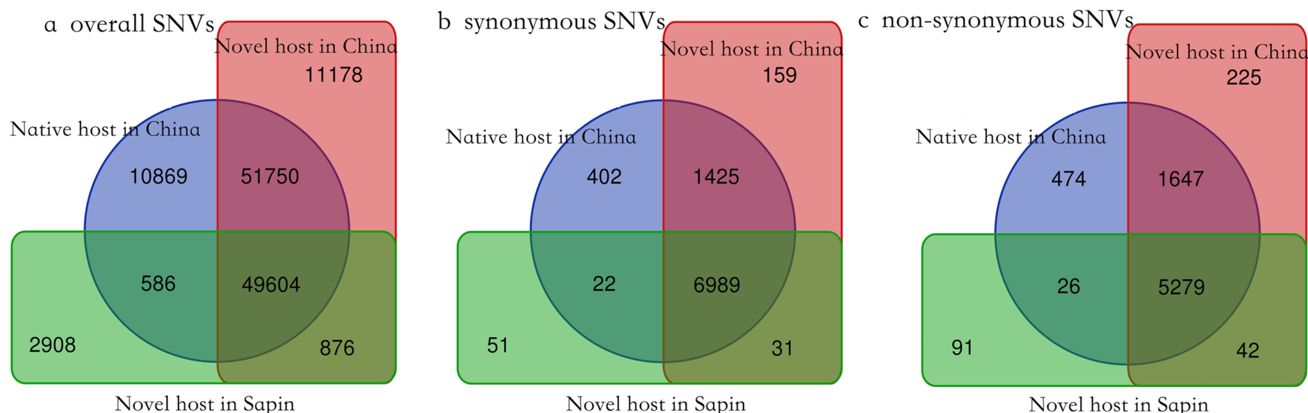

Fig. 1 Venn diagram of parasite SNVs isolated from native host and novel host in China as sympatric population, as well as novel host in Spain as parapatric populations. (**a**) Total number of SNVs, (**b**) Synonymous SNVs and (**c**) non-synonymous SNVs. Novel host in China represents the parasite isolated from the honey bee *A. mellifera* in China. Native host in China represents the parasite isolated from the honey bee *A. cerana* in China. Novel host in Spain represents the parasite isolated from the honey bee *A. mellifera* Spain

Table 1 Population genetic statistics for the three parasite isolates (Mean ± SE). π_a indicates the diversity at non-synonymous position. π_s indicates the diversity at synonymous position. *Tajima's D* was corrected for the pooled population

Parasite isolates	π_a / π_s	π	*Watterson's θ*	*Tajima's D*
Native host in China	0.2044 ± 0.0135	0.0098 ± 0.0001	0.0087 ± 0.0001	0.994 ± 0.014
Novel host in China	0.1988 ± 0.0110	0.0089 ± 0.0001	0.0074 ± 0.0001	1.447 ± 0.018
Novel host in Spain	0.1877 ± 0.0071	0.0028 ± 0.0001	0.0025 ± 0.0001	0.350 ± 0.022

loci with observed allele frequency = 1, compared with random ($P < 0.05$, Pearson's Chi-squared test). Additionally, the fixation index F_{st} was higher between the parasite isolated from parapatric populations (0.0502 ± 0.0019) than sympatric populations (0.0134 ± 0.0003) (Table 2).

Native host immune responses towards two parasite isolates from sympatric populations

Out of 39 selected immune genes, 28 were amplified in the honey bee *A. cerana* (Table S1). Out of these 28 amplified immune genes, 6 were significantly differentially expressed following the inoculation of two parasite sources from sympatric populations. Five genes were from the Toll pathway and one gene was from IMD pathway, a significant deviation from random over the four innate immune pathways in honey bees (Fisher's exact test, $P < 0.05$). The expression levels of *cactus-1* ($P < 0.05$), *defensin-1* ($P < 0.001$) and *relish* ($P < 0.05$) were significantly higher towards parasites isolated from novel host compared with those isolated from native host at a single time point. Conversely, the expression levels of *lys-1* ($P < 0.05$), *lys-2* ($P < 0.05$) and *PGRPSC4300* ($P < 0.05$) were significantly higher towards parasites isolated from native hosts compared with those isolated from novel hosts at two time points (Fig. 2). Significantly differentially expressed genes were not evenly distributed across the time points during the experimental period (Chi-square test, $P < 0.05$).

Discussion

Parasite diversity profile

As an invasive parasite, *N. ceranae* infection is reported to reduce the lifespan and suppress the immune response of the honey bee *A. mellifera* [4, 29, 38, 54]. As a novel parasite, the host might be more sensitive to the infection due to unbalanced co-evolution forces [1]. Overall, reduced parasite genetic diversity in novel hosts is expected, as a smaller subset of parasite circulate in the novel host. However, in habitats where both honey bee host species coexist, a different scenario comes into play. It is critical to determine whether parasite exchange between the novel and native hosts is ongoing in sympatric populations. Previous studies clearly revealed *N. ceranae* showed higher genetic variance within a colony than among colonies and that genetic variance among geographic locations was minor [19–21, 41, 46]. If the number of alleles of the parasite transferred to the novel host is small, the chance of minor genetic variance among host in various geographic locations is then low. Pelin and coauthors performed a genomic survey of *N. ceranae* collected from Spain, Turkey, France, Croatia, Hawaii and Argentina [41]. They found minimal variance across these widespread populations, suggesting that a majority of *N. ceranae* alleles have been transmitted to all six locations. However, alleles could still be lost at the moment when the parasite switched from the native host to the novel host [24]. Additionally, alleles could also be lost during adaptation to the novel host, while novel genotypes could arise due to recombination, countering this loss [20, 21, 41]. In this way, the genetic diversity could be actively maintained during the host-parasite co-evolution [6]. In our study, the parasites isolated from sympatric novel hosts shared a significantly higher number of SNVs compared with the native hosts versus novel hosts from parapatric populations. Our data support continuing gene flow between native and novel hosts, arguably due to commonly visited flowers in shared habitats [18, 22, 48]. As a result, the nucleotide diversity π in parasites isolated from sympatric populations were three times higher than the parapatric population. Our data also indicate that this gene flow enhances the genetic diversity of the parasite in the novel host. Overall, three isolates were indicative of purifying selection, suggested by the ratio of π_a / π_s. However, the purifying selective force may not necessarily apply to all the genes in the genome, which could still be under positive selection [5, 51]. In our data, π was slightly higher than *Watterson's θ*, leading to small positive *Tajima's D*, which may suggest a lack of low frequency alleles in all three parasite populations [17, 52]. Additionally, the lower

Table 2 Pairwise fixation index F_{st} of the three parasite isolates. The F_{st} between the parapatric populations was higher than sympatric populations

Parasite isolates	Native host in China	Novel host in China
Novel host in Spain	0.0569 ± 0.0019	0.0502 ± 0.0019
Novel host in China	0.0134 ± 0.0003	

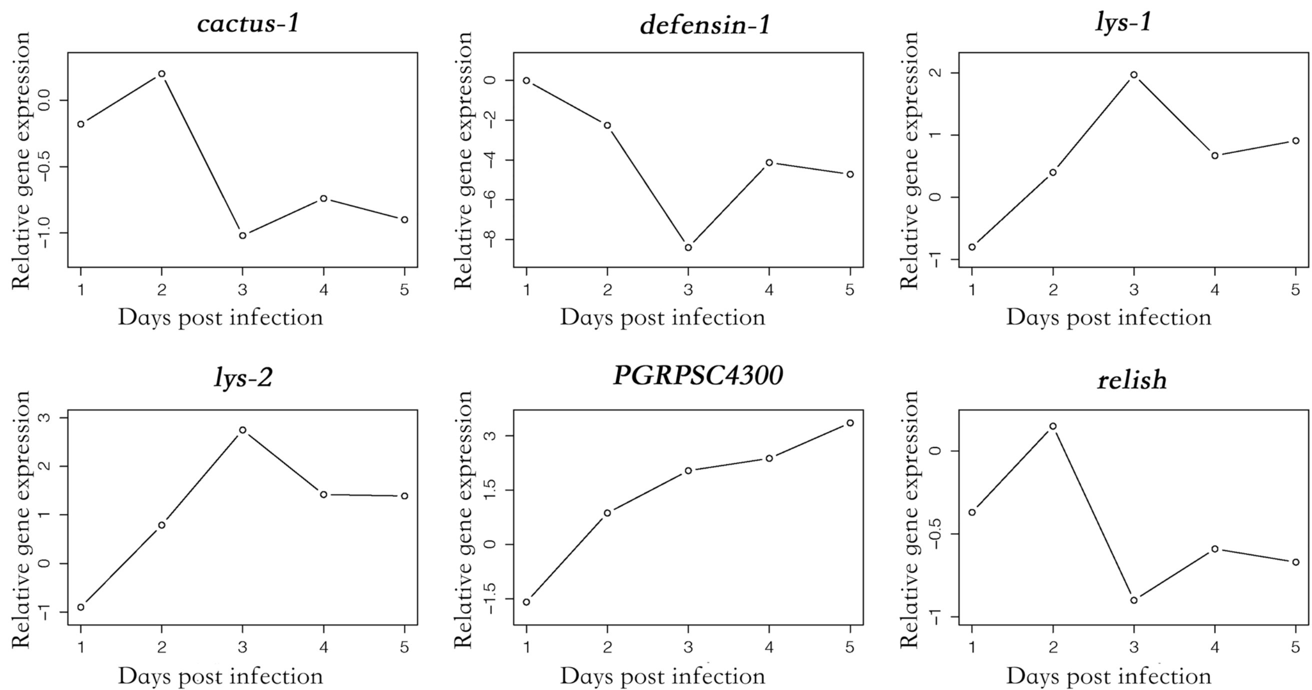

Fig. 2 Expression profile of immune genes towards *N. ceranae* spores isolated from native and novel hosts in a sympatric population. Out of 28 immune genes, 6 were significantly differentially expressed towards the two sources of parasites. Five immune genes (*cactus-1*, *defensin-1*, *lys-1*, *lys-2* and *PGRPSC4300*) were within the Toll pathway and the gene *relish* was from IMD pathway, which was significantly deviated than random ($P < 0.05$). Additionally, significant gene expression events occurred only in 3 and 5 dpi, which also deviated from random ($P < 0.05$). Y axis represents Mean expression difference triggered by parasite isolated from native host and novel host ($Mean_{native} - Mean_{novel}$). * indicates the difference is significant at $P < 0.05$ level and *** indicates the difference is significant at $P < 0.001$ level

Tajima's D value in parapatric population may suggest a population contraction compared with sympatric populations [10, 50]. In our data, the singleton sequencing error might be partially counterbalanced by high coverage reads [33]. The small value of F_{st} between the sympatric populations suggests high proportion of shared SNVs. Comparatively, a relatively larger value of F_{st} was observed between parapatric populations, suggesting that less SNVs were shared, which might be due to local adaptation [2]. Alternatively, the SNVs in Spanish population might be introduced from other region than China.

Innate immune responses

Given that parasites are exchanged between native and novel hosts, parasites are selected for adapting to novel hosts while remaining infective in native hosts [37]. By using a selected set of markers, parasites isolated from novel hosts showed more unique genotypes compared with the native hosts. Arguably, selection in novel hosts may favor virulence over transmission [14, 34, 46] As a result, host responses towards the two sources with different genotypes might be different. In our data, immune genes in the Toll pathway showed significantly different expression profiles when exposed to spores isolated from native and novel hosts. This result is consistent with previous studies in that the Toll pathway is the main innate immune response towards *N. ceranae* infection [28, 36]. In our study, significantly regulated immune genes were not identified at 4 dpi, which might reflect the time when infected epithelia cells lyse. When offspring spores were released, immune genes were activated again, as found at 5 dpi. As expression levels of the pathogen recognition receptor *PGRPSC4300* were significantly higher towards parasites isolated from native hosts compared with those from novel hosts, this suggests that adaption to the novel host influences parasite recognition [27, 45]. In another study, the expression levels of antimicrobial peptides were generally higher in native hosts compared with novel hosts following *N. ceranae* inoculation [7]. However, a consistent immune gene-expression profile has not been observed. The protein *cactus* generally inhibits immune responses while *relish* regulates the expression of antimicrobial peptides [13, 47]. In our data, the antimicrobial peptide transcripts *defensin* and *lysozyme* [13, 55] were either up or down regulated during infection. A clear pattern of native host immune responses towards the two parasite sources

was not found. Both bee and parasite genetic background could lead to differential immune responses, even though they share habitats [30, 39, 43]. Therefore, we cannot conclude that there has been any virulence selection resulting from the shared habitat so far. Our study is limited to freshly emerged bees. It would be interesting to include older bees in all seasons in a following study. Nevertheless, we can conclude that sharing the habitat across host species enhances the genetic diversity of the parasite in the novel host, since polymorphisms are continually introduced to the novel host.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-021-01827-3.

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Author contribution QH designed the experiment. QH and LK conducted the experiment. QH, LK, LZZ, WYY, ZJZ and JDE organized the manuscript.

Data availability Data generated from this study have been deposited in NCBI BioProject PRJNA602377 with accession # SAMN13893430 and SAMN19893431. Additional comparative data were downloaded from NCBI BioProject PRJNA209464 with accession # SAMN02213592.

Declarations

Competing interests The authors declare no competing interests.

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