Apidologie
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DOI: 10.1007/s13592-013-0243-4

Original article

Four quantitative trait loci associated with low *Nosema ceranae* (Microsporidia) spore load in the honeybee *Apis mellifera*

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Received 22 April 2013 – Revised 19 August 2013 – Accepted 17 September 2013

Abstract – *Nosema ceranae* has been recently introduced into the honeybee *Apis mellifera* as a novel microsporidian gut parasite. To locate the genetic region involved in *N. ceranae* infection tolerance, we fed *N. ceranae* spores to haploid drones of a F1 hybrid queen produced from a cross between a queen of a *Nosema*-resistant bred strain and drones of susceptible colonies. The spore loads of the infected F1 drones were used as the phenotype to identify quantitative trait loci (QTLs) associated with *N. ceranae* spore load. One hundred forty-eight infected drones were individually genotyped with microsatellite markers at an average marker distance of 20 cM along the genome. Four QTLs were significantly associated with low spore load, explaining 20.4 % of total spore load variance. Moreover, a candidate gene *Aubergine* (*Aub*) within the major QTL region was significantly overexpressed in drones with low spore loads than in those with high spore loads. Our results confirm the genetic basis of *Nosema* tolerance in the selected strain and show that both additive effects and epistatic interactions among the QTLs interfere with the tested phenotype.

***Apis mellifera* / drone / *Nosema* / QTL**

1. INTRODUCTION

Nosema apis and *Nosema ceranae* are two microsporidian gut parasites of the honeybee, *Apis mellifera*. *N. apis* is an evolutionarily old pathogen of the honeybee *A. mellifera* with a moderate virulence, and honeybee colonies can often cure themselves under favorable environ-

Electronic supplementary material The online version of this article (doi:10.1007/s13592-013-0243-4) contains supplementary material, which is available to authorized users.

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Manuscript editor: Peter Rosenkranz

mental conditions (Zander 1909; Chen et al. 2009). In contrast, *N. ceranae*, which was originally found in the Asian honeybee *A. cerana* (Fries et al. 1996), is a newly established parasite of *A. mellifera* (Fries et al. 2006; Higes et al. 2006). Although there were reports suggesting that *N. ceranae* had a high virulence in *A. mellifera* at both the colony and the individual level (Higes et al. 2008, 2009), more recent studies suggest a moderate virulence of *N. ceranae* similar to that of *N. apis* (Forsgren and Fries 2010; Gisder et al. 2010).

In Denmark, honeybee colonies have been selected for the absence of *Nosema* infections (*Nosemosis*) for decades (Traynor 2008). Within the Danish honeybee breeding scheme, worker samples from colonies were used to determine the *Nosema* infection level. If the samples were found infected by *Nosema*, the queen of the colony would be replaced with one reared from a colony without *Nosema* infection. Although this mode of selection was at the colony level based on the presence of *Nosema* in the worker samples, the beekeepers physically exchanged queens. This breeding scheme resulted in a honeybee strain in which *Nosema* infections are rarely found and individual bees showed a high tolerance towards experimental *N. ceranae* infections (Huang et al. 2012). Since the biological mechanisms underlying the colony level tolerance are unknown so far, the identification of associated genes (quantitative trait locus, QTL) that had been selected by the beekeepers might help to better understand the actual tolerance mechanisms.

In the honeybee, QTL mapping has been widely used to address quantitative traits and complex social behavior (Hunt et al. 1998; Oxley et al. 2010; Behrens et al. 2011). In the case of *Nosema* tolerance, we can take advantage of haploid drones for QTL mapping because the trait is also expressed in the male sex. The male haploid genetic system is particularly suited for QTL mapping studies as interpretational problems resulting from dominance interactions between the alleles on different homologous chromosomes cannot occur. We here used the drone offspring of a single hybrid queen which resulted from a cross of the queen of the *Nosema*-resistant selected Danish strain with drones of an unselected French strain to identify QTLs associated with low *N. ceranae* spore load. In a subsequent step, we quantified the expression level of a candidate gene identified in the QTL analyses in the same mapping population.

2. MATERIALS AND METHODS

2.1. Instrumental insemination of queen bees

Nine virgin queens of the selected Danish strain were provided by the Department of Integrated Pest Management Research Centre Flakkebjerg, Denmark. The queens were all artificially inseminated with the same mixed sperm (Moritz 1984) of 30 drones of an unselected strain kept at the Laboratoire de Biologie et Protection de l'abeille, INRA Avignon, France. The inseminated queens were introduced into small colonies composed of ~4,000 freshly emerged workers at the apiary of the Martin-Luther-University Halle-Wittenberg, Germany. We reared F1 hybrid queens from the inseminated queens and treated the F1 queens with CO_2 to initiate ovary activation without mating, so that the F1 queens exclusively produced unfertilized eggs developing into drones. A single F1 queen, who first started to lay eggs, was chosen to produce the mapping population. The drones were reared in drone frames in full-sized colonies.

2.2. *N. ceranae* infection

Workers freshly infected with *N. ceranae* were provided by the Laboratoire de Biologie et Protection de l'abeille, INRA Avignon France, as the source of *N. ceranae* spores for subsequent infection. The abdomens of infected workers were homogenized in distilled water, filtered through filtering paper and centrifuged at 3,220×*g* for 10 min. The pellet was re-dissolved in distilled water and centrifuged at 8,700×*g* for 5 min to purify the *N. ceranae* spores. Spores were counted using a Fuchs–Rosenthal hemocytometer and the *Nosema* species was verified by a standard PCR protocol (Hamiduzzaman et al. 2010).

Frames of sealed drone brood of the single F1 hybrid queen were kept in an incubator (34±1 °C, 60 % rel. humidity). Freshly emerged drones were collected daily from the brood frames to provide age standardized individuals (0–24 h). Freshly emerged

workers from brood frames kept in the incubator served as nurse bees. Drones were individually fed with 2 μL sucrose solution containing ~10^5 *N. ceranae* spores. Drones that did not consume the entire solution were discarded. Infected drones and uninfected nurse workers were housed in a wooden cage (depth 13.0 cm×width 10.0 cm×height 11.5 cm) at 34±1 °C and 60 % rel. humidity. Drones receiving 2 μL sucrose solution without any *Nosema* spores were kept under the same conditions. These uninfected drones served as controls for the candidate gene expression analyses. Drones and workers were fed with 50 % sucrose solution ad libitum without pollen during the remaining time of the experiment.

2.3. Drones used in the QTL analysis

In order to select the drones for the QTL mapping, ~10^5 *N. ceranae* spores were individually fed to 319 drones of the single F1 queen. All drones were sampled on day six post infection to let the *Nosema* infection spread out of the ventriculi. Dead drones were daily removed and recorded. The guts of all sampled drones were individually removed and homogenized in 500 μL distilled H_2O. Ten microliters homogenized solution was again diluted by 90 μL distilled water to count the *N. ceranae* spores in a Fuchs–Rosenthal hemocytometer. By day six post infection, 64 out of the 319 infected drones had died. It is unclear whether the 64 drones were killed by the infection or the behavior of the "nurse" workers in the cage. Since new generation *N. ceranae* spores were detected approximately 4 days after the infection (Higes et al. 2007), all drones that were dead before day four post infection (n=55) had no meaningful phenotype with relation to *Nosema* reproduction and were discarded from further analyses. The guts of the remaining 255 drones were individually removed to determine the spore load (median and range, 5.6×10^5 and 2.4×10^6). No *N. ceranae* spores were found in the control groups. 76 drones with <3.6×10^5 spores (low; median: 8×10^4, range: 3.4×10^5) and 72 drones with >8.2×10^5 spores (high; median: 1.1×10^6, range: 1.7×10^6) were selected for the QTL mapping (Figure S1). The number of spores was significantly different between these two groups (Mann–Whitney U test, $P<0.001$).

2.4. Heterozygous marker selection

After the removal of the gut, the abdomen was immediately preserved in RNA-later® (Sigma-Aldrich) and stored at −80 °C for candidate gene expression analyses. The remaining thoraces and heads were preserved in 75 % ethanol and stored at −20 °C until DNA extraction using 5 % Chelex 100 (Walsh et al. 1991) for the genotyping. We genotyped the F1 queen using 732 fluorescence-labeled microsatellite markers by multiplex PCR (according to Behrens et al. 2011) to select heterozygous markers for the individual genotyping.

2.5. Phase determination

As the mother queen of the F1 queen was accidently killed and removed by the worker honeybees, we could not use her directly to determine the phase of the used markers. We sampled ten drones each from ten colonies of the selected Danish honeybee strain, and genotyped them to determine the phase (Danish alleles and French alleles). We identified all alleles for all heterozygous loci of the selected drones. As all the drones used for the QTL mapping were from a single F1 queen with a Danish and a French allele at every locus, Danish alleles could be unambiguously identified, whenever they were unique and different from the French strain. We used these unique markers as anchor loci to determine the phase of all other loci in the mapping population based on linkage analysis.

2.6. Single QTL analysis

Drones were individually genotyped with all heterozygous makers using the MegaBACE 1000 DNA analysis system (Amersham Biosciences, Germany) and scored with the MegaBACE Fragment Profiler Version 1.2. We used the number of spores in 1 μL of the diluted gut homogenates as the mapping phenotype. Interval mapping was performed to identify the significant QTL associated with low *N. ceranae* spores using Window QTL Cartographer 2.5 (Wang et al. 2011). The statistical significance of the putative QTL was calculated with a 5,000 times permutation test walking along the genome with a genotyping error probability of 0.01. We used

interval mapping to identify major QTLs without considering interaction effects in the first instance. If a locus showed a significant association ($P<0.05$) with the spore load variance, the nearest markers flanking (up-stream and down-stream) this significant locus were used as the criteria to define a QTL candidate region.

2.7. Interaction QTLs analyses

We searched for potential paired epistatic and additive QTLs using the R/qtl package (Broman et al. 2003; R development core team 2008) with haploid diploid genome type, a genotyping error probability of 0.01 and 1 cM walking steps along the genome. All loci used for the QTL mapping were analyzed pairwise for potential additive and epistatic interactions. The statistical significance of the interactive QTLs was assessed by an analysis of variance (Bonferroni adjusted for the multiply comparison). If the interaction effects of the paired loci showed a significant association ($P<0.05$) with the spore load variance, the nearest markers flanking each of the two loci involved were used to define the two interacting QTL regions.

2.8. Candidate genes and quantitative real time PCR analysis

All open reading frames within the mapped QTL region were classified as candidate genes (NCBI, Map viewer, Amel 4.5). We used the translated amino acid sequence of each candidate gene in a BLASTX search against those of *Drosophila* and human to assess their potential biological functions.

The expression levels of a candidate gene *Aub* (primer sequence forward: 5′~TTACCAACGCCTCTCAA CCAATG~3′; reverse: 5′~AGATATACCAATTCGG CTTGACCAG~3′) was quantified using quantitative real time PCR (qPCR) in 25 drones each from the high and the low spore load group of the mapping population. Ten uninfected drones of the same F1 queen were used as controls. RNA was individually extracted from the abdomen excluding the gut similar to Huang et al. (2012). The cDNA of five drones each was pooled, resulting in five pools with high and low spore load drones each and two pools for the controls. The gene expression in the uninfected control group was used as standard to quantify the expression level of the candidate gene in response to the infection. The genes GPDH-1 and EF-1α that were not regulated by the *N. ceranae* infection were used as reference genes. The qPCR procedures and relative gene expression level analyses were carried out according to Huang et al. (2012).

3. RESULTS

3.1. Interval mapping

Of the 732 screened microsatellite markers, 216 were polymorphic and 60 anchor loci could unambiguously determine the phase of the marker alleles. The average distance of these polymorphic markers was 20 cM (Figure S2) with 90 % of the genome covered with a marker distance of less than 20 cM (99.5 % marker distance <50 cM). So the probability of missing a significant QTL was low due to the linkage gap. A total of 148 drones was individually genotyped using all 216 heterozygous markers. The linkage to the spore load was quantified for each marker by interval mapping using LOD scores. Only locus UN271 (LOD=2.5) on chromosome 14 showed a significant association with the spore load ($p<0.05$). Its nearest flanking loci K1418 and AT198 did not shown significant segregation, suggesting the candidate QTL region to be between these two loci. The significant QTL region spanned 1,598 kbp (between the locus K1418 at 5,355 kbp and AT198 at 6,953 kbp).

3.2. Fine mapping

To further narrow down the detected QTL region, we designed and genotyped the 148 drones with five additional heterozygous microsatellite markers flanking the locus UN271 in the significant QTL region (between the locus K1418 and AT198). The association between the markers and the spore load was recalculated with interval mapping. The threshold of the significant QTL region was determined by the 5,000 times permutation test which walks along the entire genome for every 1 cM. The locus UN271 (LOD=2.6) again showed a significant association with the *Nosema* spore

load, confirmed by a permutation test along the genome (LOD=2.4, P=0.05) (Figure 1). By adding additional five markers, the statistically significant QTL (QTL_m) region was narrowed down to 338 kbp, spanning from locus HQ1414 at 6,071 kbp to BI103 at 6,409 kbp explaining 7.7 % of total variance. Looking at the actual segregation of the alleles at this locus, 46 out of 76 low spore load drones had the selected Danish allele and 49 out of 72 high spore drones had the unselected French allele. This is a significantly biased allelic distribution towards the predicted phenotype (P<0.001, χ^2 test). The locus UN271 showed significant segregation, but its nearest flanking loci HQ1411 and HQ1414 did not, suggesting the candidate QTL region to be between these two loci. The frequency of the selected Danish allele decreased with the increasing number of spores (Figure S3).

3.3. Epistatic and additive effects

Locus AC184 on chromosome 3 (QTL_{ep3}) significantly interacted with the locus AT129 (QTL_{ep10}) on chromosome 10 in a two-dimensional two QTLs scan, which explained 6.3 % of the total variance (LOD=3.6, P<0.01). Drones with either the Danish or the French alleles at both loci (QTL_{ep3} and QTL_{ep10}), had a higher spore load than those with a combination of two alleles at either locus (Figure 2, Figure S4). This suggests that the interactions between the Danish allele and French allele were important for a low spore load. Additionally, we identified an additive QTL on chromosome 6 (K0616, QTL_{ad}). The additive effect between the Danish allele of QTL_{ad} and the Danish allele of the major QTL_m was also associated with a low spore load explaining 6.4 % of the total variance (LOD=2.5, P<0.05) (Figure S4).

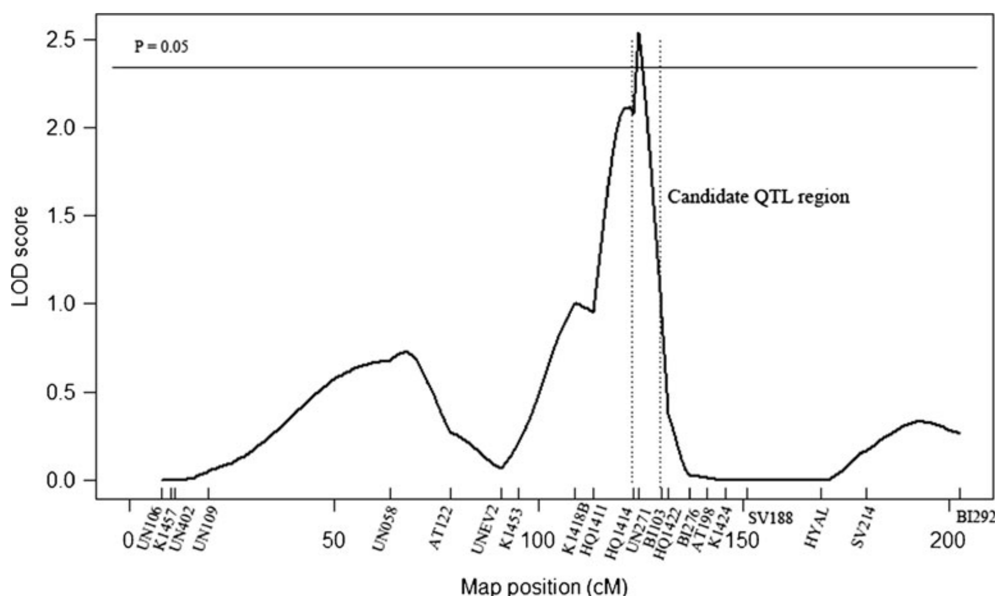

Figure 1. Significant QTL on Chromosome 14. The threshold for the significant QTL was calculated by 5,000 time permutation test along the genome with the genotyping error probability of 1 %. The QTL is statistically significant at P=0.05 level with LOD score of 2.4. By interval mapping, the marker UN271 showed a significant association with the *Nosema* spore load (LOD=2.6). The significant QTL region was located between the marker HQ1414 and BI103 (spanning 338 kbp) explaining 7.7 % of the total variance. The locus UN271 showed significant segregation, but its neighboring loci HQ1411 and HQ1414 did not, suggesting the target region is between these two loci. We used the interval between these two neighboring markers to search the candidate genes (represented by the *vertical dashed line*).

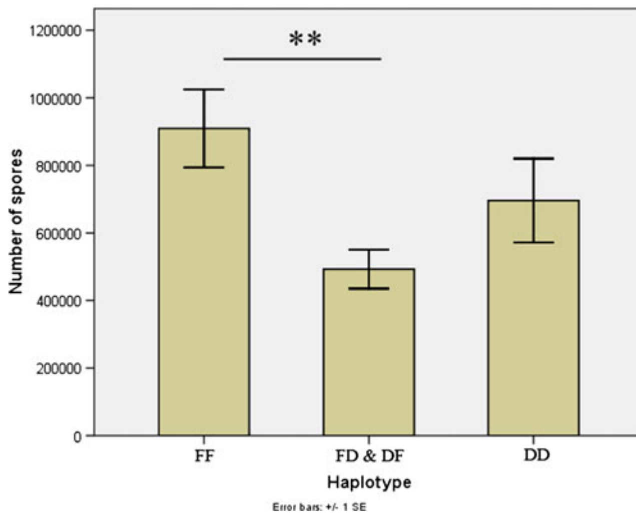

Figure 2. Observed spore load for two-locus genotype groups at the location of two epistatically interactive QTLs. AC184 which is in chromosome 3 significantly interacted with AT129 on chromosome 10 by explaining 6.3 % of total variance. D and F represent the allele originated from Danish and French strains respectively. The genotype "DD" represents the Danish allele at both AC184 in chromosome 3 and AT129 in chromosome 10. The genotype "DF" represents the Danish allele at AC184 and French allele at AT129. Drones with the genotype of "DF" and "FD" had significantly lower (P=0.002) spore load than the drones with "FF" allele at both loci which suggests the direction of the epistatic effect is to low spore load. **Represents the significant level at P<0.01, ANOVA, two-tailed test, Bonferroni adjusted for the multiply comparison.

From the actual allelic segregation, 86 % of the drones with a low spore load carried at least one Danish allele at either locus of QTL_{ad} or QTL_m; 79 % of the drones that carried the Danish alleles at both loci showed a low spore load, which was a strong and significant bias compared to the random combination of two alleles towards the predicted phenotype (P<0.01, χ^2 test).

3.4. Candidate genes and expression profiles

We searched for candidate genes within the QTL_m region (between the loci HQ1414 and BI103) and QTL_{ad} (between the loci K0618 and 5388); 31 and 11 genes respectively could be identified (Table S1, Table S2). However, the gene *Aubergine* (*Aub*) within QTL_m was of particular interest. *Aub* is a member of the *Argonaut* family containing the typical active catalytic piwi domain, which functions as a nucleic acid binding domain and is involved in RNA interference (Kawaoka et al. 2008; Liao et al. 2010). The expression level of *Aub* was upregulated after *N. ceranae* infections in drones with both high and low spore load in comparison to the controls. Hence, *Nosema* infection enhanced the *Aub* expression. This effect was however stronger in the low than high spore load group. *Aub* was significantly more overexpressed in drones with a low spore load than in those with a high spore load (two-tailed t test, P<0.05) (Figure 3).

4. DISCUSSION

4.1. One major QTL on chromosome 14

We could confirm previous results that this breeding strain shows a strong tolerance towards the *Nosema* infections (Huang et al. 2012). The genetic basis of this trait is also reflected by the coefficient of variance for spore load within the selected (c_v=0.37) and unselected population (c_v=0.43) (Huang et al. 2012),

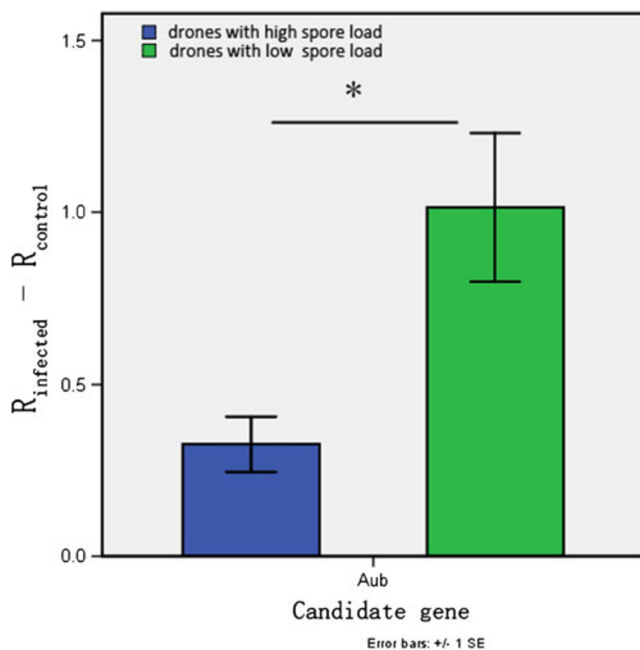

Figure 3. Candidate gene expression profiles between drones with high spore load and low spore load. The *Y*-axis is the relatively gene expression value. R_{infected} and R_{control} represent the relative gene expression value of the infected drones and control drones respectively. Comparing with the controls, the expression level of *Aub* was upregulated toward *N. ceranae* infection in drones with both high and low spore load. *Aub* was significantly higher expressed in drones with low spore load than drones with high spore load. *Significant level, $P<0.05$, two-tailed *t* test.

compared to the coefficient of spore load variance in the QTL mapping population (c_v=0.88) of this study. We here found four QTLs to be significantly associated with the *Nosema* spore load. With the average marker distance of ~20 cM, the power to identify a QTL explaining 7 % of the total variance exceeds 95 % (Rebai et al. 1995). Although QTLs with small effects might remain undetected, the chance is low that we have missed QTLs with larger effect than the identified significant QTLs. The QTL on chromosome 14 might play a major role (major QTL=QTL_m) for the low number of *Nosema* spores in the selected Danish honeybees, since it explained 7.7 % of the total variance. Even though the Danish allele of QTL_{ad} on chromosome 6 showed a significant association with the low spore load, this effect only occurred together with the Danish allele of the major QTL_m. Nevertheless, the combination of both loci might help to implement marker assisted breeding in the Danish population. Indeed a selective sweep could be detected in the QTL_m region (Huang et al. in revision) in the breeding population that independently confirms the importance of this locus for the tolerant phenotype.

4.2. Epistatic effects

In addition to the additive gene effects also the epistatic interactions between loci on chromosomes 3 and 10 interfered with the spore load. In this case, it was the combination of Danish and French alleles that caused a low spore load. Epistatic interactions had also been involved in *Varroa destructor* resistance (Behrens et al. 2011). In our case, the epistatic

interactions cannot be used for selective breeding because it is very likely impossible to maintain the specific allele combination at the two loci to conserve the same epistatic effects in a breeding line. Nevertheless, this example showed again the power of using drones for QTL studies as it is very likely we would have missed any epistatic effects if we had used diploid workers instead.

4.3. Candidate gene

Even though the *Aub* was the prominent candidate gene, we can of course not exclude that also other candidate genes might be involved in the tolerance towards *N. ceranae* infection. From a functional perspective, *Aub* is of particular interest, as it is involved in regulating via RNA interference machinery known to interfere with foreign RNA (Liao et al. 2010; Teo et al. 2011). In *Drosophila*, *Aub* has been shown to be involved in the silencing of retrotransposons via RNA interference (Kawaoka et al. 2008). Moreover, it was reported to be involved in the resistance towards the gut bacterium *Serratia marcescens* infection in the *Drosophila* (Cronin et al. 2009). A reduction in *Aub* transcript levels in *Drosophila* resulted in an increase of mortality due to bacterial infection. This fits to our findings of increased transcript levels in low spore load drones. Unfortunately, we do not know the exact mechanisms in which *Aub* is involved in the context of tolerance towards *N. ceranae* infections. *Aub* may trigger an apoptosis of the infected cells in order to prevent the production of spores, potentially with the help of immune genes. Indeed six innate immune genes from three different immune pathways had significantly higher expression levels in drones of the selected strain that in those of unselected strain (Huang et al. 2012). We also found the expression level of *Aub* to be higher in the selected strain, albeit not statistically significant which might be due to a low sample size (unpublished data). Nevertheless, this might reflect a general enhanced immune response of bees of the selected strain against *Nosema* infections which may have considerably contributed to the selection success of the Danish bee breeders.

ACKNOWLEDGMENTS

We would like to thank Dieter Behrens and Petra Leibe for technical assistance; Holger Scharpenberg and Hans-Hinrich Kaatz for the assistance with bee keeping; Didier Crauser for the artificial insemination. This work was supported by the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement BEE DOC FP7-KBBE-2009-3 244956CP-FP, the Deutsche Forschungsgemeinschaft Project DFG SPP 1399(MO 373/26-1) and the China Scholarship Council.

Quatre locus quantitatifs associés à une faible charge de spores de *Nosema ceranae* (Microsporidia) chez l'abeille *Apis mellifera*

***Apis mellifera* / mâle / *Nosema* / QTL**

Vier QTL die mit einer geringen Sporenbelastung von *Nosema ceranae* (Microsporidia) bei Honigbienen (*Apis mellifera*) assoziiert sind

***Apis mellifera* / Drohnen / *Nosema* / QTL**

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