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# A selective sweep in a microsporidian parasite *Nosema*-tolerant honeybee population, *Apis mellifera*

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**Summary**

*Nosema* is a microsporidian parasite of the honeybee, which infects the epithelial cells of the gut. In Denmark, honeybee colonies have been selectively bred for the absence of *Nosema* over decades, resulting in a breeding line that is tolerant toward *Nosema* infections. As the tolerance toward the *Nosema* infection is a result of artificial selection, we screened chromosome 14 for a selective sweep with microsatellite markers, where a major quantitative trait locus (QTL) had been identified to be involved in the reduction in *Nosema* spores in the honeybees. By comparing the genetic variability of 10 colonies of the selected honeybee strain with a population sample from 22 unselected colonies, a selective sweep was revealed within the previously identified QTL region. The genetic variability of the swept loci was not only reduced in relation to the flanking markers on chromosome 14 within the selected strain but also significantly reduced compared with the same region in the unselected honeybees. This confirmed the results of the previous QTL mapping for reduced *Nosema* infections. The success of the selective breeding may have driven the selective sweep found in our study.

**Keywords** honeybee, genetic diversity, *Nosema*, resistance, selective breeding

## Introduction

*Nosema* is a microsporidian parasite of the honeybee (*Apis mellifera*), which infects the epithelial cells of the gut (Zander 1909). *Nosema* lives as an obligate intracellular parasite. The infection starts from the ingestion of the spores, which germinates in the midgut and extrudes the polar tubes that penetrate the epithelial cells to release the sporoplasm into the cytoplasm (Higes *et al.* 2007; Fries 2010). The infected cells eventually will burst and release a new generation of spores (de Graaf *et al.* 1994; Gisder *et al.* 2011). The offspring spores either can germinate to infect new host cells or might be expelled through the feces (Gisder *et al.* 2011).

*Nosema* infection affects honeybees in multiple ways (Haseman 1951, 1952; Huang *et al.* 2012) including increased mortality (Mayack & Naug 2009). Two *Nosema* species are known to infect the honeybee *A. mellifera*: *N. apis* and *N. ceranae*. *N. apis* is an evolutionarily old pathogen of *A. mellifera* with a moderate virulence. The host–parasite coevolutionary relationship is well balanced, and colonies often can cure themselves under favorable environmental conditions (Zander 1909; Chen *et al.* 2009). *Nosema ceranae* originally was found in the Asian honeybee *A. cerana* (Fries *et al.* 1996) and is a newly established parasite of *A. mellifera* (Fries *et al.* 2006; Higes *et al.* 2006). Although it has been reported to have caused large colony losses (Higes *et al.* 2008, 2009), there are increasing reports of a moderate virulence of *N. ceranae* similar to that of *N. apis* (Forsgren & Fries 2010; Fries 2010; Gisder *et al.* 2010).

Irrespective of its virulence, *Nosema* adds to the pathogen load in honeybee colonies and reduces their productivity (Kralj & Fuchs 2010). This is why bee keepers in Denmark

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embarked on a selection program aiming at *Nosema* absence since the 1980s (Traynor 2008). Originally, 500 colonies (*A. mellifera*) were involved in the selective breeding, reflecting an effective population size of about $n_e = 1846$ (Kerr 1975; Owen & Owen 1989) given each queen is mated with an average of about 12 males (Schlüns *et al.* 2005). Queens were naturally mated on an island, and the colonies were checked every year for *Nosema* infections. The queens were replaced with queens from *Nosema* absent colonies whenever the workers were infected by *Nosema*. Hence, selection was performed at the colony level without knowing which biological mechanism drove *Nosema* absence in the sampled workers. Nevertheless, this selective breeding should have left 'footprints' of selection in the Danish breeding population whether one or a few major genes determined the free of infection phenotype. If a specific beneficial allele was selected for, this allele might eventually become fixed in the breeding population, known as a hard sweep or classic selective sweep (Palaisa *et al.* 2004; Hermisson & Pennings 2005). Neighboring neutral markers closely linked to the locus under selection also should show reduced variability because of genetic hitchhiking (Maynard Smith & Haigh 1974; Charlesworth *et al.* 1993; Chevin & Hospital 2008). Alternatively, the bee breeders might have removed a susceptibility allele (or alleles) from the population. In this case, more than one allele might be selected for, and the genetic variability of the surrounding neutral loci should also be reduced, known as soft sweep (Hermisson & Pennings 2005).

In the honeybee, selection can operate at two different levels: the individual level and the colony level. The phenotype of the colony may not just reflect the genotype of the queen but rather the combined effect of all colony members based on the genotypic composition of the entire colony (Moritz 1986). Hence, detecting *Nosema*-infected workers might be due to the individual genotype but might also be due to the genotypic composition of the entire colony. In spite of these genetic complications, Huang *et al.* (in press) detected a major quantitative trait locus (QTL) on chromosome 14, which was associated with the reduction in the *Nosema* spores in experimentally infected individual haploid male bees. This made it a promising candidate QTL region that might be responsible for the *Nosema*-tolerant phenotype at the colony level. This QTL region (6071–6409 kbp on chromosome 14, Amel 4.5) was identified by individually genotyping a mapping population of 148 drones with 221 heterozygous microsatellite markers spanning the full genome. If this QTL is responsible for the breeding success, then positive selection might have generated a selective sweep in this region given the very recent selection events. Here, we used microsatellite markers within and flanking the identified QTL region to screen for a reduction in genetic variability in the same selected Danish honeybee strain providing the mapping population for the QTL identification. Moreover, we compared the genetic variability in this region with an unselected honeybee population to analyze the potential selective sweep (Kim & Stephan 2002; Nielsen *et al.* 2005).

## Materials and methods

### Drones collection and DNA extraction

Ten drones per colony were collected from 10 hives from the Danish selected population in Slagelse, Denmark. Moreover, 32 drones were collected from a non-selected control population at a drone congregation area (DCA) in Halle, Germany. Sampling drones on a DCA remove the bias of sampling specific breeding lines at apiaries, because the drones originate from many different apiaries surrounding the DCA in a range of 2 km (Kraus *et al.* 2005). Hence, the population sample is less apiary biased and better reflects an unselected population that might even include feral colonies. The Danish sample could not be sampled in the same way because we explicitly needed to have a sample of the specific breeding strain. All the drones were stored in 75% ethanol at −20 °C until the DNA was extracted using 5% Chelex-100 (Bio-Rad; Walsh *et al.* 1991).

### Genotyping

The drone genotypes were used to infer the mother queen genotypes. These queen alleles were used to determine the allele frequencies of the population. The colonies of the unselected control population contributing drones of the DCA were reconstructed using the maximum likelihood algorithm of COLONY 1.3 (Wang 2004) to avoid an estimation bias due to sampling drones with the same chromosomal set that originated from the same mother queen. Ten fluorescence-labeled microsatellite markers within (*UN271* and *K1452*) and flanking (*UNEV2, K1453, BI116, K1418B, AT198, K1424, SV188* and *HYAL*) the QTL region were used to assess the genetic variability (Table 1). Additionally, six randomly chosen and unlinked fluorescence-labeled microsatellite markers on chromosomes 1, 3, 6, 8 and 10 served as reference loci to estimate the background genetic diversity of the two populations (Table 1). Each multiplex PCR contained 1 μl DNA (50 ng/μl), 5 μl master mix (Promega) and 0.4 μl/primer (10 mM, 6–8 primers per reaction), adding water for a final volume of 10 μl. The PCR cycle was as follows: 95 °C for 15 s; 55 °C for 30 s; and 72 °C for 30 s for 40 cycles. The allele sizes were determined using MegaBace 1000 capillary DNA sequencer (Amersham Biosciences) and scored with MegaBACE Fragment Profiler version 1.2.

### Population genetic analysis

To detect the presence of a selective sweep, we estimated the expected heterozygosity ($H_e$) for each locus based on the

**Table 1** Locus, chromosome, physical position, expected heterozygosity and the number of effective alleles for the 16 genotyped loci.

| Reference loci | Ch. | Kb | $H_{eS}$ | $A_{eS}$ | $H_{eC}$ | $A_{eC}$ | lnR$\theta$ | Mean ± SD | |
|---|---|---|---|---|---|---|---|---|---|
| *AC127* | 1 | 1,109 | 0.72 | 3.52 | 0.69 | 3.22 | 0.12 | 1.09 ± 1.01 | |
| *6701* | 3 | 9,693 | 0.77 | 4.42 | 0.80 | 4.92 | −0.22 | | |
| *K0616* | 6 | 11,669 | 0.52 | 2.09 | 0.74 | 3.90 | −1.44 | | |
| *K0808* | 8 | 4,417 | 0.49 | 1.96 | 0.83 | 5.80 | −2.44 | | |
| *AT168* | 8 | 5,381 | 0.65 | 2.88 | 0.86 | 7.27 | −1.96 | | |
| *AT129* | 10 | 11,106 | 0.39 | 1.65 | 0.52 | 2.07 | −0.64 | | |
| Tested loci | Ch. | Kb | $H_{eS}$ | $A_{eS}$ | $H_{eC}$ | $A_{eC}$ | lnR$\theta$ | *T* value | Original *P* value | Adjusted *P* value |
| *UNEV2* | 14 | 4,466 | 0.51 | 2.02 | 0.70 | 3.30 | −1.17 | 0.06 | 0.48 | >0.05 |
| *K1453* | 14 | 4,684 | 0.63 | 2.68 | 0.46 | 1.85 | 0.94 | −1.58 | 0.91 | >0.05 |
| *BI116* | 14 | 4,825 | 0.65 | 2.90 | 0.89 | 8.83 | −2.34 | 0.96 | 0.19 | >0.05 |
| *K1418B* | 14 | 5,355 | 0.35 | 1.54 | 0.80 | 4.95 | −2.83 | 1.34 | 0.11 | >0.05 |
| *UN271* | 14 | 6,124 | 0.10 | 1.11 | 0.72 | 3.53 | −3.89 | 2.17 | 0.04 | >0.05 |
| *K1452* | 14 | 6,265 | 0.10 | 1.11 | 0.91 | 11.5 | −6.33 | 4.06 | 0.0049 | <0.05* |
| *AT198* | 14 | 6,953 | 0.10 | 1.11 | 0.88 | 8.12 | −5.62 | 3.51 | 0.0085 | >0.05 |
| *K1424* | 14 | 7,174 | 0.53 | 2.11 | 0.53 | 2.13 | −0.02 | −0.83 | 0.78 | >0.05 |
| *SV188* | 14 | 7,432 | 0.64 | 2.75 | 0.52 | 2.08 | 0.68 | −1.38 | 0.89 | >0.05 |
| *HYAL* | 14 | 8,319 | 0.52 | 2.09 | 0.37 | 1.59 | 0.78 | −1.45 | 0.90 | >0.05 |

Kb, physical position on the chromosome in Kbp (based on Amel 4.5); $H_{(eS),}$ expected heterozygosity in the selected population; $A_{(eS),}$ number of effective alleles in the selected population; $H_{(eC),}$ expected heterozygosity in the unselected control population; $A_{(eC),}$ number of effective alleles in the unselected control population; and lnR$\theta$, natural logarithm transformation of the ratio between the two populations.
*$P < 0.05$, Bonferroni adjusted for 10 comparisons, one tailed *t*-test.

reconstructed queen genotypes and corrected for the sample size (Alam *et al.* 2011) as follows:

$$H_e = \frac{n}{n-1}(1 - \sum p_i^2),$$

where $n$ = the number of sampled chromosomes and $p_i = i^{th}$ allele frequency at a locus.

In addition, we determined the number of effective alleles ($A_e$) for the given locus (Nagylaki 1985) as follows:

$$A_e = 1/(1 - H_e),$$

where $H_e$ = the expected heterozygosity of a locus.

To account for the initial population differences and locus-specific effects on the pattern of genetic diversity (low mutation rate, selective sweep or background selection), we used a control population that had not been exposed to the artificial selection as a comparison. The population variation estimator ($\theta = 4\mu N_e$) of each locus was calculated according to Schlötterer (2002) and Kauer *et al.* (2003), assuming that the marker loci in both populations had the same mutation rate. We used the natural logarithm transformation of the ratio (lnR$\theta$) between the two populations to indicate the genetic diversity difference for each locus (Ohta & Kimura 1973; Wiehe *et al.* 2007) as follows:

$$\text{lnR}\theta = \ln \frac{\left(\frac{1}{1-H_{\text{selected}}}\right)^2 - 1}{\left(\frac{1}{1-H_{\text{control}}}\right)^2 - 1},$$

where $H_{\text{selected}}$ = expected heterozygosity of the selected population and $H_{\text{control}}$ = expected heterozygosity of the unselected control population.

As lnR$\theta$ of unlinked loci have been shown to follow a normal distribution (Kauer *et al.* 2003; Wiehe *et al.* 2007), we compared the lnR$\theta$ of the reference loci with each of the 10 target loci on chromosome 14 using a *t*-test. The *t* value of 10 tested loci on chromosome 14 was calculated to indicate the existence of the selective sweep as follows:

$$t = \frac{\text{Mean}_1 - \text{Mean}_2}{\sqrt{\left(\frac{N_1 * S^2 + N_2 * S^2}{N_1 + N_2 - 2}\right) * \left(\frac{1}{N_1} + \frac{1}{N_2}\right)}},$$

where $t = t$ value of each locus in chromosome 14, $\text{Mean}_1$ = mean lnR$\theta$ value of reference loci, $\text{Mean}_2$ = lnR$\theta$ value of each locus in chromosome 14, $N_1$ = number of lnR$\theta$ value of reference loci, $N_2$ = number of lnR$\theta$ value of the tested loci and $S$ = standard deviation of reference loci.

## Results

### Expected heterozygosity

In the selected population, the genotypes of 10 queens were successfully reconstructed. Because drones develop from the unfertilized eggs of the queen, they carry only one maternal chromosomal copy comprising the QTL and the entire linkage group under study. In a set of 10 drones per colony, the allelic composition at the series of the 10 linked loci at both of the queens' chromosomes can be reconstructed with a probability of 99.8% for the two entire linkage groups (Table S1). In all cases, both maternal alleles were detected in each set of 10 drones. By calculating the relatedness among drones of the control population, 29 sets of

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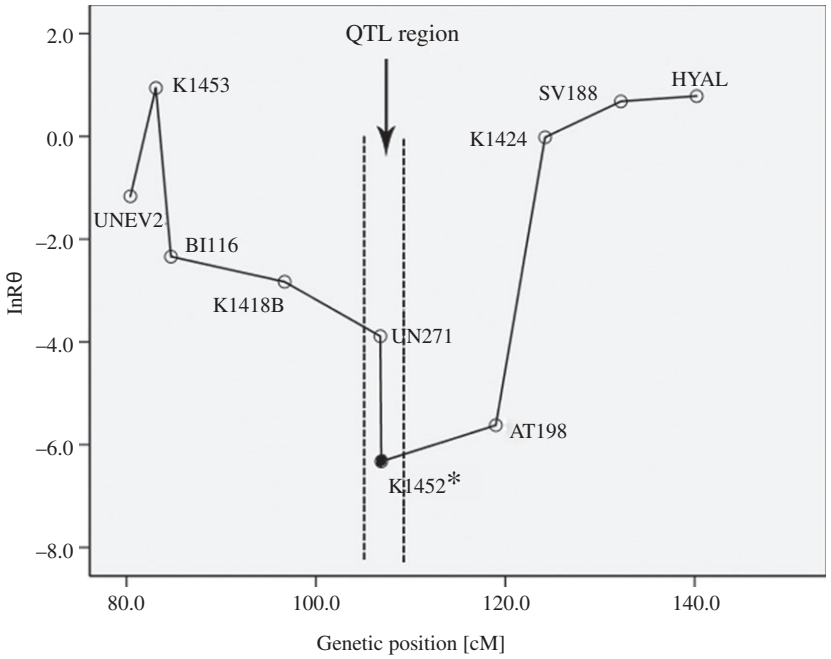


**Figure 1** The lnR$\theta$ between the selected and unselected control populations on chromosome 14. The locus *K1452* was swept due to the drastically reduced genetic variability in the selected population in relation to both randomly chosen marker loci and the same loci in the unselected population. The previously mapped quantitative trait locus region for reduced number of *Nosema* spores showed a selective sweep. *$P < 0.05$, Bonferroni adjusted for 10 comparisons.

chromosomes were constructed from 32 drones that were classified into $22 \pm 1.1$ colonies (Table S1). The allele frequencies were calculated based on the 29 sets of chromosomes (Table S1) to avoid an estimation bias due to sampling drones with the same chromosomal set that originated from the same mother queen. The loci *UN271, K1452* and *AT198* had the same lowest genetic variability in the selected population. The expected heterozygosity was $H_e = 0.1$ for these three loci, which was much lower than the seven neighboring flanking loci ($H_e = 0.55 \pm 0.04$) and the six reference loci ($H_e = 0.58 \pm 0.05$). In the control population, these three loci had a higher average expected heterozygosity ($H_e = 0.84 \pm 0.06$) than did the neighboring ($H_e = 0.61 \pm 0.07$) and reference loci ($H_e = 0.74 \pm 0.05$). By comparing the two populations, the expected heterozygosity was most strongly reduced at the loci *UN271*, *K1452* and *AT198*, whereas the neighboring and reference loci were similar (Table 1).

## The number of effective alleles

To visualize the potential allele fixation, we compared the number of effective alleles ($A_e$) of the two populations. The loci *UN271, K1452* and *AT198* showed the lowest number of effective alleles of $A_e = 1.11$ in the selected population that was almost fixed. The average number of effective alleles of the seven flanking loci was more than twice as high ($A_e = 2.3 \pm 0.18$) but slightly lower than the six reference loci ($A_e = 2.8 \pm 0.43$). In the unselected population, the locus *K1452* had the highest number of effective alleles with $A_e = 11.5$. The loci *UN271, K1452* and *AT198* had a high average number of effective alleles ($A_e = 7.72 \pm 0.23$), which was even higher than the neighboring ($A_e = 3.5 \pm 0.99$) and the reference loci ($A_e = 4.5 \pm 0.76$) in the selected population (Table 1).

## Selective sweep

To calculate the $t$ value to detect the existence of the selective sweep, we compared the six reference loci ($-1.09 \pm 1.01$, mean $\pm$ SD) with each of 10 target locus with a two-sample $t$-test by assuming equal variance. The locus *K1452* showed a significantly lower genetic diversity in the selected population than did the control population compared with the reference loci ($df = 5$, $t = 4.06$, one-tailed $t$-test, $P < 0.05$; Bonferroni adjusted for 10 comparisons), which gave evidence for the existence of the selective sweep to reduce *Nosema* infection (Fig. 1 and Table 1). To obtain confidence limits testing the power of the six reference loci, we used a jack-knifing resampling approach over loci. Thus, the mean and standard deviation of five reference loci was calculated six times, leaving out one locus each. Testing this jack-knifed overall mean ($-1.0873 \pm 1.0108$) vs. the swept locus *K1542* confirmed the selective sweep of the locus *K1452* ($df = 4$, $t = 3.88$, $P < 0.01$, one-tailed $t$-test). Furthermore, if the sweep was

false positive due to a monomorphic locus in the initial breeding population, the diversity of the neighboring loci should not have been affected. A regression of the diversity ($\ln R\theta$) of seven adjacent loci on the genetic distance (cM) to the target locus yielded $R^2 = 0.7$ ($P = 0.015$), which gave additional support for the swept region.

## Discussion

The low expected heterozygosity and the low number of effective alleles clearly revealed that the genetic diversity in the QTL region for *Nosema* tolerance was strongly reduced in the selected honeybee population. The comparison with an unselected control population revealed a selective sweep in the same genomic region that was associated with the reduction in *Nosema* spores in the selected honeybee population. The statistic method we used to detect the selective sweep was based on Kauer *et al.* (2003). They used a large marker set spanning two chromosomes including the loci under selection as reference loci, because in their case, the candidate region under selection was unknown. We used fewer reference loci because we already had information on a candidate sweep region from a QTL mapping study. The genetic variance was most strongly reduced spanning the three loci (*UN271, K1452* and *AT198*) in the selected population. When we compared the genetic variance with the same loci in the control population, the locus *K1452* showed the highest reduction in both genetic diversity and effective alleles (more than an order of magnitude).

If locus *K1424* reflects the true sweep, the reduced diversities of loci *UN271* and *AT198* could be a result of hitchhiking with the actual locus under selection (Fay & Wu 2000). Surprisingly, the locus *K1424* was heterozygous in all queens of the selected strain. But the effective number of alleles and expected heterozygosity were similar between the selected and unselected population. Based on our current knowledge on pedigree information, we have no other plausible explanation for the consistent heterozygosity other than chance. It seems very unlikely that we found a genetic system similar to the genetic load at the sex locus with lethal selection against homozygotes (Mackensen 1951, 1955). Given that the selected allele detected in the previous QTL study is close to fixation, it appears that the classic selective sweep has driven the success of breeding programs at the colony level. As we do not have the genetic information about the initial breeding population, we cannot unambiguously conclude whether this selected allele is from the standing variation or a new mutation.

The low levels of heterozygosity obviously resulted from the low number of alleles in the selected population. The selective breeding dramatically decreased the number of effective alleles at locus *K1425* from 11.5 in the unselected population to 1.1 in the selected population. This extreme reduction clearly is more likely a result of a hard sweep rather than a soft sweep (Chevin & Hospital 2008; Stephan 2010). Even though a soft sweep can decrease the genetic diversity of the selected and flanking loci, it is unlikely to lead to fixation (Charlesworth *et al.* 1993; Charlesworth 1996).

After over 20 years of selective breeding, the number of alleles of reference loci in the breeding population is smaller but not significantly different from the unselected population (*t*-test, $P > 0.05$). Because we included both neighboring loci and a set of unlinked reference loci to detect the selective sweep, we provided two independent sets of information to show that selection rather than random drift caused the reduction in the number of alleles in the sweep region. Given the high recombination rate (19 cM/Mb) in the honeybee genome (The Honeybee Genome Sequencing Consortium 2006), the selective sweep in the selective Danish honeybee population must have occurred extremely fast. Otherwise, the recombination should have eroded the trace footprints of selection before the markers linked to the selected locus became monomorphic (Moritz & Evans 2008). A similar case of extreme positive selection has been reported for various species including vector mosquitoes for malaria disease. The mosquitoes quickly became resistant to insecticide treatment, and the genomic region controlling drug resistance could be identified by a selective sweep (Lynd *et al.* 2010; Norris & Norris 2011).

In our study, the selection was conducted at the colony level, but it showed clear footprints of positive selection at the individual level. So the simple procedure of replacing queens in susceptible colonies with queens from colonies which lacked *Nosema* left this selective footprint in the population structure. The colony level selection has selected a locus that confers tolerance against *Nosema* to individual bees. The effect of a major QTL on the individual bee was identified through the reduction in the *Nosema* spores in guts of individual bees. Experimentally infected selected bees also showed a significantly higher tolerance toward *Nosema* infection than did the unselected control bees (Huang *et al.* 2012). The results presented in this study revealed a selective sweep as a result of colony level selection (Palaisa *et al.* 2004; Chevin & Hospital 2008), with the swept loci and the QTL in the same genomic region. Hence, two studies (individual level QTL mapping and the colony level selective sweep analysis) used different approaches but obtained the same result, which provides strong support to the notion that the identified genetic region is indeed associated with the tolerance toward the *Nosema* infection.

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## Supporting information

Additional supporting information may be found in the online version of this article.

**Table S1** Queen genotypes of the selected and the control honeybee populations.