The genetic basis of natural variation in a phoretic behavior

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Phoresy is a widespread form of commensalism that facilitates dispersal of one species through an association with a more mobile second species. Dauer larvae of the nematode Caenorhabditis elegans exhibit a phoretic behavior called nictation, which could enable interactions with animals such as isopods or snails. Here, we show that natural C. elegans isolates differ in nictation. We use quantitative behavioral assays and linkage mapping to identify a genetic locus (nict-1) that mediates the phoretic interaction with terrestrial isopods. The nict-1 locus contains a Piwi-interacting small RNA (piRNA) cluster; we observe that the Piwi Argonaute PRG-1 is involved in the regulation of nictation. Additionally, this locus underlies a trade-off between offspring production and dispersal. Variation in the nict-1 locus contributes directly to differences in association between nematodes and terrestrial isopods in a laboratory assay. In summary, the piRNA-rich nict-1 locus could define a novel mechanism underlying phoretic interactions.
Species dispersal has been an important topic in evolutionary biology since Charles Darwin’s era. He was fascinated by how one species facilitates the migration of another species. For example, he described a phoretic interaction between ducks and freshwater snails after observing just-hatched snails attached to a duck’s foot, suggesting a dispersal mechanism for the wide-range distribution of snails. Since that time, numerous reports of phoresy have accumulated, but the genetic bases of these interactions remain elusive. To address the genetic underpinnings of a natural phoretic interaction, we investigated the association between the stress-resistant, long-lived dauer larvae of the nematode *Caenorhabditis elegans* and terrestrial isopods.

Interspecific association and phoretic dispersal of dauer larvae are conserved among *Caenorhabditis* species (Supplementary Movie 1), and this interaction is facilitated by a dauer-specific phoretic behavior called nictation where dauer larvae lift and wave their bodies, presumably to increase interactions with more mobile species.

Because natural variants are known to contribute to phenotypic differences in wild populations, quantitative genetic approaches have been used to successfully elucidate the molecular bases of the natural variation underlying a variety of *C. elegans* traits. We investigated natural variation in the nictation behavior of *C. elegans* wild isolates, utilizing a previously established quantitative assay to measure the fraction of nictating dauer larvae from a population of moving dauers for a collection of genetically diverse wild strains (Fig. 1a and Supplementary Data 1). We found that 12 strains differed significantly (one-way analysis of variance (ANOVA), \( p < 0.001 \)) in nictation behaviors over a fourfold range, indicating that natural strains likely have diverse phoretic interactions mediated by nictation. We found that a smaller fraction of CB4856 dauers nictate than dauers of the N2 strain (Fig. 1a), and recombinant inbred lines have been constructed to enable linkage-mapping approaches. Using this mapping approach, we identified a quantitative trait locus (QTL) on chromosome IV that contributes to variation in nictation behavior. This locus contains a large cluster of Piwi-interacting small RNAs (piRNAs), which are all regulated by the Piwi Argonaute PRG-1. Because we could not perturb single piRNAs out of many hundreds, we generated *prg-1* mutant strains to test the causal connection of piRNAs to nictation behavior. These results show that the N2 strain contains piRNAs that likely inhibit genes that mediate nictation. We go on to demonstrate that this QTL underlies a phoretic behavior with terrestrial isopods and contributes to a potential fitness trade-off.

### Results

#### Natural variation in nictation behavior

To establish whether natural strains of *C. elegans* vary in a potential dispersal behavior, we focused on nictation—a behavior where the long-lived dauer larvae lift and wave their bodies to increase interactions with larger, more mobile species. We used a previously established quantitative assay to measure the fraction of nictating dauer larvae among a population of moving dauers for a collection of genetically diverse wild strains (Fig. 1a and Supplementary Data 1). We found that 12 strains differed significantly (one-way analysis of variance (ANOVA), \( p < 0.001 \)) in nictation behaviors over a fourfold range, indicating that natural strains likely have diverse phoretic interactions mediated by nictation. We found that a smaller fraction of CB4856 dauers nictate than dauers of the N2 strain (Fig. 1a), and recombinant inbred lines have been constructed to enable linkage-mapping approaches using these two strains. Additionally, N2 dauers show the fifth highest mean nictation fraction out of the 12 strains measured, indicating that differences between these two strains fall within the range of natural behavioral variation.

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**Fig. 1** *C. elegans* differ in a phoretic behavior map to chromosome IV. **a** The mean nictation fractions of 12 divergent wild isolates are shown (from left, CB4856 (blue), EG4725, ED3017, JT11389, N2 (orange), JU258, JU775, DL238, LKC34, CX11314, MY16, and MY23). Twenty-four replicates of CB4856 and three replicates of all other strains were measured to calculate the means. Error bars are standard deviations. **b** A histogram of the normalized nictation fractions of 186 N2×CB4856 recombinant inbred advanced intercross lines (RIALs) is shown. **c** Linkage-mapping results of normalized nictation fraction are shown with genomic position (Mb) on the x-axis and logarithm of odds (LOD) score on the y-axis. The tick marks on the x-axis denote every 5 Mb. Each chromosome is in its own box labeled on top. The gray line is the LOD threshold for 5% genome-wide error rate obtained by permuting the phenotype data and mapping 1000 times. The red triangle denotes the peak QTL marker. **d** Tukey box plots of N2 (orange) and CB4856 (blue) are shown with data points plotted behind. The horizontal line in the middle of the box is the median, and the box denotes the 25th to 75th quantiles of the data. The vertical line represents the 1.5 interquartile range.
Identification and fine mapping of the nict-1 QTL. We sought the genetic causes underlying this difference in nictation by measuring the behaviors of 186 recombinant inbred advanced intercross lines (RIAILs) derived from a cross between N2 and CB4856 (Fig. 1b and Supplementary Data 2). Linkage mapping revealed a single significant QTL, which we named nict-1, on the right arm of chromosome IV (Fig. 1c). This locus does not overlap any of laboratory-derived loci, including npr-1, glp-5, and nath-10, that were discovered previously. Recombinant strains with the N2 nict-1 genotype exhibited a higher nictation ratio compared to those strains with the CB4856 genotype (Fig. 1d), consistent with the parental difference.
To validate and to narrow the nict-1 QTL, we generated near-isogenic lines (NILs) by crossing the nict-1 QTL genomic region from N2 into the CB4856 genetic background, as well as performing the reciprocal cross. The phenotypes of these NIL strains confirmed the QTL effect in both genetic backgrounds, with the N2 nict-1 interval promoting higher nictation than the CB4856 nict-1 interval (Fig. 2a, b and Supplementary Data 3). By generating recombinants across the confirmed nict-1 region, we created nine additional NILs containing smaller N2 genomic regions in the CB4856 background. The differences in nictation behaviors of these NILs narrowed the nict-1 QTL to a 73 kb region (Fig. 2a, b and Supplementary Data 3).

**piRNAs contribute to differences in nictation behavior.** The nict-1 QTL genomic region contains four protein-coding genes, three pseudogenes, two non-coding RNA genes, and 289 annotated 21U piRNAs genes (Fig. 2c). Using characterized sequence variation between the two parental strains N2 and CB4856, we found that only one (Y105C5A.1272) of the four protein-coding genes contained variation between the N2 and CB4856 strains. The variant in Y105C5A.1272 encodes a putative serine-to-glycine change that could alter gene function (Supplementary Data 4). Additionally, we investigated gene expression of the four protein-coding genes and the three putative pseudogenes by quantitative RT-PCR. We found that the N2 strain expresses Y105C5A.1272 gene, whereas the CB4856 strain does not (Supplementary Data 5). We tested two independently generated deletion alleles of this gene in the N2 nict-1 genetic background (Supplementary Fig. 2). Although each deletion removes a large proportion of the Y105C5A.1272 coding sequence, the two mutant strains did not show the same effect on nictation (Supplementary Fig. 1 and Supplementary Data 6), suggesting that Y105C5A.1272 does not underlie differences in nictation between the two strains. Of the other three protein-coding genes in the interval (Fig. 2c), we found that Y105C5A.15 had a detectable expression difference between the parental strains and the nict-1 NILs (Supplementary Data 5).

To test whether this gene expression difference could cause the nictation effect, we assayed a deletion allele of Y105C5A.15 in the N2 background and found that it does not have an effect on nictation (Supplementary Figs. 1 and 2 and Supplementary Data 6), suggesting that none of the protein-coding genes in the nict-1 interval play a role in nictation differences between the N2 and CB4856 strains.

Next, we investigated variation in pseudogene sequences and expression in both the N2 and CB4856 strains. We found that the three pseudogenes have variants that would eliminate gene function and remain pseudogenes in both genetic backgrounds. Additionally, expression of these three pseudogenes did not correlate with the differences in the nict-1 QTL genotype (Supplementary Data 5). These results led us to consider the 289 21U piRNA genes. Because specific perturbations of individual piRNA genes often do not cause loss of gene regulation and the 289 piRNA genes are distributed throughout the 73-kb nict-1 QTL region, single gene perturbations are not feasible. However, the more than 12,000 21U piRNA genes, including those genes in this region, require the Piwi Argonaute encoding gene, prg-1 (Fig. 2c). Therefore, we globally perturbed piRNA functions by deleting exons one through seven of the prg-1 gene in both the N2 and CB4856 genetic backgrounds using the CRISPR/Cas9 genome-editing system. Loss of prg-1 in the N2 genetic background resulted in a nictation fraction similar to the CB4856 strain (Fig. 2d). Furthermore, loss of prg-1 in the CB4856 genetic background had a similar nictation fraction as the CB4856 strain, implicating N2-specific piRNAs in the control of this phoretic behavior. Additionally, an independent prg-1 loss-of-function allele in the N2 genetic background had a similar effect on nictation (Supplementary Fig. 3 and Supplementary Data 6). These small RNA genes regulate the expression of transposons and also endogenous genes. Any one or multiple of these targeted genes could play a role in the regulation of nictation behavior. Because expression of prg-1 is restricted to the germline, it remains elusive how differences in piRNAs can underlie differences in nictation behaviors.

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**Fig. 3** The nict-1 QTL controls a fitness trade-off and species dispersal on isopods. 

- **a** Brood sizes of N2, CB4856, LJ1203 (nict-1 N2 > CB4856) are shown as Tukey box plots colored based on the nict-1 genotype. The horizontal line is the median, and the box denotes the 25th to 75th quantiles of the data. The vertical line represents the 1.5 interquartile range. All four strains were scored in 30 biological replicates, except for LJ1213 where only 29 biological replicates were scored. 

- **b** The mean N2 nict-1 QTL allele frequencies before (origin) and after (destination) transmission competition assays are plotted. Error bars are standard deviations. 

- **c** The experimental scheme of the transmission competition assay is shown. Transmission competition assay chambers contain two nematode culture plates. The "origin" plate contained a mixture of dauer animals from the CB4856 and LJ1213 (nict-1 N2 > CB4856) strains and was covered with medical gauze to facilitate nictation. The "destination" plate was placed 1-2 cm away with no nematodes on this plate. Terrestrial isopods were added to the chamber and allowed to roam freely for 24 h. Nematodes depend on the isopods for transfer to the "destination" plate from the "origin" plate because no transfer was observed without isopods presence. Eleven different biological replicates were scored.
**nict-1 controls a trade-off between nictation and reproduction.** The *nict-1* QTL likely contributes to different fitness consequences for wild *C. elegans* strains. The NIL strains had brood sizes that were significantly different from their respective parental genetic backgrounds (Fig. 3a and Supplementary Data 7). The offspring production of *nict-1* from N2 crossed into the CB4856 background (LJ1213) was lower than that of the CB4856 parent, and the brood size of *nict-1* from CB4856 crossed into the N2 background (LJ1203) was higher than N2. Therefore, the *nict-1* QTL likely confers a trade-off between nictation and reproduction with the N2 version, promoting nictation but inhibiting offspring production. This result implies that the N2 strain has a functional piRNA that acts to promote nictation and the same or an independent piRNA that acts to inhibit reproduction. Alternatively, a linked N2 variant in a protein-coding gene could exert an opposing effect on reproduction in the N2 *nict-1* genotype. Next, we analyzed a correlation between the nictation fraction and previously determined offspring production of the wild strains (Supplementary Fig. 4). We did not find a strong correlation between these two traits (*r* = −0.1666), suggesting that the trade-off between nictation and reproduction might be specific to the *nict-1* QTL between the N2 and CB4856 strains.

**nict-1** underlies phoretic dispersal mediated by isopods. Natural populations of *C. elegans* exhibit local genetic diversity 30, suggesting that intraspecific competition occurs among wild strains in the natural environment. To examine how quantitative variation in nictation behavior relates to the dispersal of *C. elegans* in a competitive environment, we developed a transmission competition assay to quantitatively analyze isopod phoresy (Fig. 3b, c). To mimic this natural phoretic interaction, we used the terrestrial isopod, *Porcellio scaber*, so that we can test whether *C. elegans* can hitchhike to a more favorable environment. Isopods were previously reported as a natural carrier of *C. elegans* 31, and dauers can readily attach to them. We induced dauers from two different *nict-1* genotypes in the same culture to determine whether the N2 *nict-1* genotype, which confers a higher nictation fraction, could confer a higher transmission frequency than the CB4856 *nict-1* genotype. Because of differences in offspring production caused by the trade-off discussed above, the culture contained four times as many dauers of the CB4856 *nict-1* genotype than the N2 *nict-1* genotype (Fig. 3b and Supplementary Data 8). Despite this disparity on the origin plate, we found that dauers with the N2 *nict-1* genotype were transported to the destination plate at a rate four times higher than the rate of the CB4856 *nict-1* genotype. This transport was dependent on the presence of terrestrial isopods. These results suggest that variation in *nict-1* underlies this natural phoretic interaction. Furthermore, given the potential intraspecific competition in the natural environment, our results demonstrate that the *nict-1* QTL could control the ability of strains to colonize new bacteria-rich environments.

**Discussion**

We propose that the *nict-1* QTL controls a hitchhiking behavior and phoretic dispersal, as well as a trade-off between dispersal and reproduction, two traits necessary for the survival and evolution of the *C. elegans* species. Behavioral diversity arises from the gene–environment interface where a biological system confronts an ever-changing natural niche, and multiple genes are involved in such dynamic interactions 32. Although this study identifies a novel difference between the laboratory strain and a wild strain, the observed difference in nictation does not appear to be laboratory-derived because the N2 strain is not exceptional in this behavior, as it is for other traits optimized in the laboratory 33. Our study provides strong evidence that the nictation behavior is controlled by differences in regulatory small RNAs, which could act via a large number of distributed effects to create robust environmental adaptations 34. Involvement of the piRNA pathway in neuronal plasticity is suggested from studies of the *Aplysia* central nervous system and the mammalian brain, implying conserved regulatory roles among metazoans 35,36.

Studies of a large collection of diverse wild strains are required to understand the broad applicability of the piRNA-rich *nict-1* QTL effect across the *C. elegans* population. This proposed molecular mechanism of behavioral control enables future studies of this phoretic interaction and its precise genetic causes across *C. elegans* and related nematode species.

**Methods**

**Wild isolates.** CB4856, CX1314, DL238, ED3017, EG4725, J11398, JU238a, JU775, KLC34, M16Y, M232, and N2. Strain data including isolation location, isolate information, and more are available from CeNDR (https://www.elegansvariation.org) 37.

**RAILs.** QX2-QX17, QX19, QX20, QX22, QX24-QX27, QX29, QX32-QX34, QX35-QX37, QX47-QX49, QX50, QX91-QX96, QX102-QX104, QX106-QX118, QX120-QX122, QX124, QX125, QX127-QX129, QX131, QX134, QX137, QX138, QX140, QX147, QX148, QX153, QX154, QX156-QX166, QX169-QX178, QX180-QX193, QX195-QX198, QX200-QX207, QX212, QX213, QX216-QX221, QX224-QX227, QX229-QX237, and QX239.

**NILs.** LJ1203 snuIR3 (*nict-1* QTL, CB4856 > N2), LJ1204 snuIR4 (*nict-1* QTL, N2 > CB4856), LJ1205 snuIR5 (*nict-1* QTL, N2 > CB4856), LJ1207 snuIR7 (*nict-1* QTL, N2 > CB4856), LJ1209 snuIR9 (*nict-1* QTL, N2 > CB4856), LJ1210 snuIR10 (*nict-1* QTL, N2 > CB4856), LJ1211 snuIR11 (*nict-1* QTL, N2 > CB4856), LJ1212 snuIR12 (*nict-1* QTL, N2 > CB4856), LJ1213 snuIR13 (*nict-1* QTL, N2 > CB4856), LJ1215 snuIR15 (*nict-1* QTL, N2 > CB4856).

Mutants. FX6979 Y105CSA.1272(tm6979), FX1161 Y105CSA.15(mn161), ECA286 prg-1(n4357) (backcrossed to the Andersen lab 20 times), ECAS54 prg-1(ed28), ECAS56 prg-1(ed30), LJ1216 snuIR13 (*nict-1* QTL, N2 > CB4856), Y105CSA.1272(mn33).

**Dauer induction and nictation assays.** Ten to twenty L4 larvae or young adults were transferred to synthetic phenrome plates containing agar (10 g/L), agarose (7 g/L), NaCl (2 g/L), KH2PO4 (3 g/L), K2HPO4 (0.5 g/L), cholesterol (8 mg/L), and synthetic phenrome-ascoroside 1, 2, 3 (2 mg/L each) 38, 39, seeded with *Escherichia coli* OP50 at 25°C for dauer induction 40. After 4 days, dauers were morphologically identified by their dark intestines and radially constricted bodies. Dauer chips were made by pouring 3.5% agar solution onto poly-dimethylsiloxane (PDMS) mold 41. Solidified agar-micro-dirt chip was detached from the PDMS mold and dried for 90 min at 37°C. More than 30 dauers were collected by glass capillary (Kimble Chase Life Science and Research Products LLC) using M9 buffer and mounted on a micro-dirt chip. After 10–30 min, when dauers were actively moving, a fraction of nictating dauers among moving dauers on a micro-dirt chip was measured three times consecutively, and the mean value of these technical replicates was represented as the nictation fraction for that biological replicate. The mean nictation fraction was calculated from the independent biological replicates of multiple nictation assays. We calculated the normalized nictation value by fitting a linear model, nictation fraction = assay date. Number of independent biological replicates for each assay is described in figure legends.

**Quantitative genetic analyses.** The nictation fractions of 186 RAIL strains from an advanced intercross between N2 and CB4856 were scored as described above. The phenotype data and genotype data were entered into R and scaled to have a mean of zero and a variance of one for linkage analysis. QTLs were detected by calculating logarithm of odds (LOD) scores for each marker and each trait as −ln(1−r2)/2ln(10), where *r* is the Pearson correlation coefficient between RAIL genotypes at the marker and phenotype trait values 42, 43. We randomly permuted the phenotype values of each RAIL while maintaining correlation structure among phenotypes 10 times to estimate empirical LOD score. The 95% confidence intervals were defined as the regions contained within a 1.5 LOD drop from the maximum LOD score.

**NIL construction.** LJ1203 snuIR3 (*nict-1* QTL, CB4856 > N2) was made from QX4, a RAIL with the CB4856 *nict-1* QTL interval on an otherwise N2 chromosome IV. QX4 was backcrossed to the N2 parent six times while selecting for the CB4856 phenotype (single-nucleotide polymorphism (SNP) genotyping). LJ1201 snuIR4 (*nict-1* QTL, N2 > CB4856) was made from QX162, a RAIL with the N2 *nict-1* QTL interval in an otherwise CB4856 chromosome IV. QX162 was backcrossed to...
the CB4856 parent six times while selecting for the N2 nict-1 QTL by SNP genotyping. To genotype the nict-1 QTL interval determined by linkage mapping (15.57–16.01 Mb), we used a Dral restriction fragment length polymorphism (RFLP) to genotype a SNP at 15,370,599 (left, marker haw66786) and 16,004,711 (right, marker haw65332). Other NLLs were generated by backcrossing LJ1204 to CB4856 further. Precise introgression breakpoints of each NLL were determined by genotyping SNP markers at position described in Supplementary Table 1 using the primers described in Supplementary Table 2.

Data availability. All data are available in Supplementary Data 1–8.

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Author contributions

D.L., E.C.A., and J.L. designed experiments, analyzed and interpreted the data, and wrote the manuscript. H.Y. performed the nictation assay for 12 wild isolates and J.K. performed the brood size assay. S.C.B. and S.Z. generated the prg-1 CRISPR/cas9 deletion alleles. M.Z. analyzed genome variation and backcrossed the prg-1(w357) allele. H.K. and Y.P. supplied synthetic phenotypes. L.K. edited the manuscript.

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