Variation in social feeding behavior and interactions among *Caenorhabditis* nematodes

Haskell D[1], Palo J[1], Eugene RFH[1], Large CRL[1], Hart MP[1]

Affiliations
[1]Department of Genetics, Perelman School of Medicine, University of Pennsylvania
* corresponding author Michael P. Hart – email: hartmic@pennmedicine.upenn.edu

Abstract

The ability to respond to complex stimuli and environmental cues is essential for organisms to survive and reproduce. Responding to a wide range of stimuli requires a neuronal network that can integrate cues and execute behavioral responses. Evolution of behaviors occurs ubiquitously in most established ecological niches, even among closely related species. To uncover the genetic and neuronal drivers of evolving behaviors, we have taken advantage of the large and relatively ancient divergence in the *Caenorhabditis* genus to ask how different *Caenorhabditis* nematodes respond to environmental stimuli and whether behavioral traits are shared or distinct. Here, we assayed foraging behaviors of twelve members of the *Caenorhabditis* clade, including members of both the *elegans* and *japonica* supergroup, and the basal taxon *C. monodelphis*. For each species, we analyzed social feeding and bordering behaviors, which are well characterized in *C. elegans.* These behaviors are the functional readout of complex sensory integration of multiple sensory cues including pheromones, touch, O_2/CO_2 concentration, and attractive and noxious stimuli. We hypothesized that the evolutionary divergence between species would correlate to divergence in these behaviors. We observed a wide variation in social aggregate feeding and bordering behaviors of hermaphrodite and female animals, but the variation did not correlate with evolutionary relatedness of the species. Addition of male animals with female or hermaphrodite animals of the same species increased aggregation behavior of subset of species, but not others. Combination of a second species with *C. elegans* significantly reduced aggregate feeding behavior of *C. elegans*, but not the other species. Intraspecies and interspecies interactions therefore modifies behavioral paradigms. Overall, we find that foraging and social feeding behaviors vary widely across *Caenorhabditis* species, likely due to species-specific responses and integration of environmental and context sensory cues. In general, the clade represents a compelling model to dissect evolution of behavior across diverse environments and a large timescale.

87 **Introduction**

88 Behavior is how organisms interact with their environment and respond to both internal
89 and external stimuli. Simple forms of life, especially those that represent unicellular phyla, rely
90 heavily on the detection of chemical and tactile stimuli through direct contact[1]. In contrast, broad
91 sensory specialization is heavily emphasized in Eukaryotes, which range in the complexity of their
92 specialization (including the evolution of differentiated and highly specialized neuronal tissues)
93 but also in the manner and breadth of their behavioral responses to the environment[2,3]. The
94 evolution of complex behavior can be loosely correlated with the evolution of multicellularity and
95 complex tissues[4]. Likewise, tissue differentiation also gave rise to neural tissues, which in their
96 simplest form, link networks of sensory cells together forming a basic neural circuit. In more
97 complex Eukaryotes, more sophisticated neural networks, utilizing multiple sensory and neuronal
98 cell types form the basis of complex organs such as neural ganglion, nerve tracts, and the brain[5,6]

99 Changes in behavioral traits occurs regularly in most eukaryotic organisms, especially
100 between related species[7]. Logically, rapid shifts in behavior should be tightly coupled with the
101 congruent alteration of their physiological and genetic drivers as highlighted by several studies in
102 *Drosophila* and related species[8,9]. Small changes in gene regulatory networks, modest alterations
103 in neural circuits, or mutations influencing receptor/ligand interactions are all sufficient to alter
104 behavioral paradigms in an often-unpredictable manner. Without the ability to identify stepwise
105 drivers of shifting behavioral paradigms it can be challenging to understand how selective
106 pressures lead to modification of underlying genetic and molecular factors, which ultimately
107 dictate the neuronal activity underlying complex responses to stimuli.

108 Compounding the study of behavioral evolution is the complex nature of behavior itself.
109 To truly differentiate between behavioral paradigms requires dissection at multiple levels of
110 organismal complexity, starting with changes to the genome and epigenome, and culminating in
111 intra-circuit alterations leading to modifications in the complex network of integrated and often
112 co-dependent behaviors. While comprehensive dissection of genetic and molecular drivers of
113 altered or novel behaviors is rarely achieved, we can utilize existing genetic information in clades
114 such as *Caenorhabditis* to inform research to begin circuit level descriptions.

115 Amongst common lab models, the nematode *Caenorhabditis elegans* is a particularly
116 powerful tool for studying neuronal circuits and behaviors. In addition to the many genetic tools,
117 a fully mapped neuronal connectome (including chemical, electrical, and peptide signaling),

118 provides a powerful model to link stereotyped behaviors with alterations of neuronal activity at
119 both a single cell and circuit level. In addition, the *Caenorhabditis* clade of nematodes represents
120 a unique opportunity to study the evolution of behavior in a diverse and evolutionarily ancient
121 lineage **(Figure 1)**[10]. Nematodes of this clade are found worldwide, with populations found in
122 nearly every ecological niche probed for their presence[11]. In the wild, members of the
123 *Caenorhabditis* nematodes persist in a free-living state generally colonizing bacteria-rich
124 substrates, such as rotting fruit, allowing them to utilize the local microbiota as a food source.
125 Considering their global dispersion, individual members of the clade experience significant
126 variation in local environment, including differences in temperature, humidity, concentration of
127 oxygen, and dominant microbiota[12]. Notably, these species are rarely isolated from homogenous
128 populations; for example *elegans* is frequently co-isolated with *briggsae* and *remanei* from the
129 same substrate. Altogether, the *Caenorhabditis* clade presents a compelling model through which
130 to query the evolution of behavior across a large evolutionary timescale and a wide range of
131 environments.

132 Due to high levels of variation among local environments, we chose to first assay foraging
133 behaviors, which we reasoned would be ubiquitously present among the clade given likely similar
134 or shared feeding strategies. One of the most well-characterized feeding behaviors is termed social
135 feeding and is defined by social interactions that occur between individuals feeding in the same
136 local environment[13]. Although not well defined, one can speculate that this behavior has costs and
137 benefits for individual animals and for populations. Animals may be attracted to high density food
138 that is resource rich for obvious nutrient seeking reasons, but animals populating a food/bacteria
139 rich location can also result in spreading of the food source (bacteria) to surrounding areas. Further,
140 group feeding could facilitate short-distance signaling between animals (perhaps through
141 pheromones) and/or increase opportunities for mating, but it seems clear that this behavior is
142 environment and context dependent[14,15]. Early surveys of wild *elegans* isolates showed a
143 surprisingly high level of variability in social feeding behaviors. Contrary to the widely used N2
144 Bristol lab strain, which displays solitary feeding, many wild isolates from a range of environments
145 show higher levels of social feeding[13]. de Bono & Bargmann defined the solitary feeding behavior
146 of N2 Bristol strain to a gain of function missense mutation in the *npr-1* gene (ortholog of
147 neuropeptide Y receptor)[13]. This results in an amino acid change in the third intracellular loop of
148 the NPR-1 G-protein coupled receptor. Loss of function mutations in the *npr-1* gene result in social

149 feeding in *elegans*[13]. For the purpose of this study we utilize the *npr-1(ad609)* loss of function
150 mutant to induce social feeding in the N2 lab control strain, which represents a reliable and robust
151 positive control for *elegans* social feeding behaviors observed in wild isolate *elegans* strains[16].
152 Subsequent work further elucidated the role of NPR-1 and other neuronal drivers of solitary
153 and social feeding, including that *elegans* social feeding behavior is driven primarily through the
154 integration of environmental cues including pheromones, O_2/CO_2 concentrations, and perception
155 of touch and noxious stimuli [14,17–20]. Sensory neurons including ADL, ASH, ASK, ADE and AWB
156 form a circuit to detect the local sensory environment which is then integrated primarily through
157 the interneuron RMG to alter locomotion paradigms[17,18]. Genetic dissection of this sensory circuit
158 has uncovered distinct roles for the neuropeptide receptor NPR-1[13,16] and gap junction connections
159 between involved sensory neurons and the RMG interneuron[17,21]. More recent work has implicated
160 glutamate signaling from ADL and ASH sensory neurons, where conserved synaptic adhesion
161 molecules (NRX-1 and NLG-1) contribute to social feeding, and *nrx-1* impacts architecture and
162 function of ASH chemical synaptic connections[16]. Therefore, social feeding behavior relies on co-
163 dependent signaling of neuropeptides, and electrical and chemical synapses, which together fine-
164 tune the balance between solitary and social feeding behaviors[16]. Given the complexity of this
165 behavioral circuit, we asked whether social feeding behaviors are conserved throughout the
166 *Caenorhabditis* clade of nematodes.
167 In this study, we compared social feeding behaviors of twelve *Caenorhabditis* species to
168 multiple strains of *elegans* (**Figure 1**). This clade of nematodes is not only relatively ancient, but
169 also has significant divergence over evolutionary time, with an estimated genomic divergence of
170 > 20 million years between *elegans* and the four most-closely related species[22]. Two large
171 subgroups, *elegans* and *japonica*, have emerged based on genomic data providing multiple
172 lineages to interrogate[10]. *monodelphis*, representing the basal taxon of the clade, is estimated to
173 have diverged over 100 million years ago, an evolutionary timescale far beyond that of the split
174 between mice and humans [23]. Lastly, the true strength of this model lies in the opportunity to study
175 "wild" populations of nematodes (representing both hermaphroditic and gonochoristic species) in
176 a controlled lab setting applicable to medium-throughput behavioral assays. When considering the
177 evolution of behavior within the *Caenorhabditis* clade we set out to compare differences amongst
178 the species, providing a base on which future work investigating differences in sensory cues,
179 neuronal function, and circuitry can be launched.

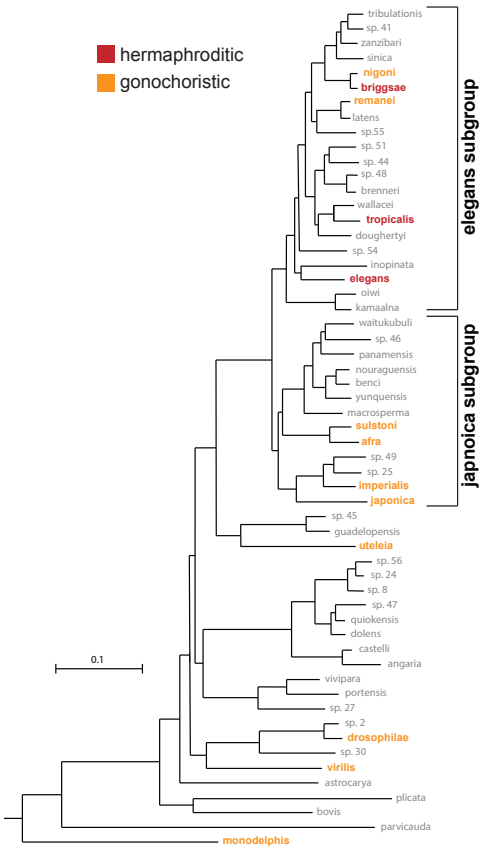


Figure 1. The *Caenorhabditis* clade of nematodes is an evolutionary and diverse lineage. (A) Phylogenetic tree of *Caenorhabditis* clade showing the two large subgroups (*elegans* and *japonica*) and the basal taxon *monodelphis*. Species included in behavioral assays are highlighted and color-coded based on reproductive strategy (red = hermaphroditic or orange = gonochoristic). Figure was adapted from Stevens et al., 2020[10].

Using the twelve members of the *Caenorhabditis* clade we found significant variation in social feeding behaviors of hermaphrodites and females amongst the different species, with all but two showing reduced levels of aggregate feeding compared to the *elegans* social feeding control strain, and half of the species significantly increasing social feeding than the *elegans* solitary control strain. Furthermore, by testing more ecologically relevant populations in our assays we identified the impact of intraspecies and interspecies interactions. Mixing males with females (or hermaphrodites) of individual species altered aggregate feeding behavior in two species when compared to homogeneous female (or hermaphrodite) populations. Combining a second species with social feeding *elegans* was sufficient to significantly reduce aggregation feeding of *elegans*. This work represents the first characterization of foraging and social feeding behaviors across the

197 *Caenorhabditis* clade of nematodes and lays the groundwork for the dissection of ecological,
198 cellular and circuit level mechanisms of conserved behaviors.
199
200 **Table 1. Reference table for *Caenorhabditis* species information.**

Species	Subgroup	Strain	*RS	*GH	Genome Accession	*MC	Behavioral Characterization
elegans	*elegans*		⚥	lab strain	PRJNA13758	yes	yes
N2							
CB4856							
briggsae	*elegans*	AF16	⚥	lab strain	PRJNA10731	yes	yes
imperialis	*elegans*	EG5942	MF	30x inbred	GCA_963572205.1	n/a	no
nigoni	*elegans*	JU1422	MF	25x inbred	PRJNA384657	n/a	no
remanei	*elegans*	PB4641	MF	20x inbred	PRJNA577507	yes	some
tropicalis	*elegans*	JU1373	⚥	n/a	PRJNA53597	n/a	no
japonica	*japonica*	DF5081	MF	20x inbred	GCA_963572235.1	yes	no
afra	*japonica*	JU1286	MF	20x inbred	GCA_963570955.1	n/a	no
sulstoni	*japonica*	JU2788	MF	25x inbred	PRJEB12601	n/a	no
virilis	*drosophilae*	JJU1968	MF	25x inbred	GCA_964036255.1	n/a	no
drosophilae	*drosophilae*	DF5112	MF	20x inbred	PRJEB67483	n/a	no
uteleia	*guadalopensis*	JU2585	MF	25x inbred	GCA_963573275.1	n/a	no
monodelphis	n/a	JU1667	MF	20x inbred	GCA_964197825.1	yes	no

*RS - Reproductive Strategy; ⚥ represents hermaphroditic species, MF represents gonochoristic species

* GS- Genetic Heterogeneity; number of times inbred according to CGC strain information

*MC - Morphological Characterization

201
202 **Methods**
203 **Strains Maintenance**
204 Animals were maintained under normal growth conditions (~20° C) on normal NGM media[24].
205 Plates were seeded with *E. coli* OP50 to provide food. Strains utilized in this study are listed in
206 **Table 1**. *npr-1*(*ad609*) and *nrx-1*(*wy778*) mutations were confirmed in *elegans* strains and were

207 used as positive controls. All other species were obtained from the CGC and maintained by
208 chunking.
209
210 **Social feeding behavior assay**
211 Standard 6-well culture plates were filled with 6 mLs of standard NGM media and then seeded
212 with 75 µL of *E. coli* OP50 and allowed to dry overnight. For the standard assay, 50 animals were
213 picked onto clean plates and then moved onto the seeded wells, transferring as little bacteria as
214 possible to prevent buildup. For standard social feeding and bordering assays (**Figure 2B&C**) the
215 50 animals were either female or hermaphrodites depending on the species. For the mixed sex
216 assays, we combined 25 males with 25 females/hermaphrodites (depending on species).
217 Regardless of the composition of the assay, worms were picked during and placed on the assay
218 during their fourth larval stage. Female or hermaphrodite animals were staged based on the
219 presence of half-moon shape characteristic of a L4 developing vulva. Males were staged based on
220 relative size, germline features, and morphological development of the tail. After placing the
221 animals on the 6-well plate, a small amount of Tween20 was used to thinly coat the lid to prevent
222 condensation forming during imaging. The plates are imaged using the WormWatcher automated
223 imaging platform (Tau Scientific) for a total of 20 hours with a cluster of 10 images (over 1 minute
224 timecourse) being taken every hour. To quantify social feeding behavior each well was manually
225 scored from blinded images, where a worm was considered aggregating if it was contacting two
226 or more other worms[13,16]. Bordering was assayed by calculating the percentage of solitary animals
227 in direct contact with the border region of the bacterial lawn (with the border defined as
228 approximately half a body length in width). Data shown is from the 15-hour mark, which placed
229 the animals of all species in the middle of Day 1 of adulthood.
230
231 **Mixed species assays**
232 In order to assay the effect of mixed species, we first generated a fluorescently tagged
233 *elegans* strain to differentiate them from the other species while imaging behavior. *npr-1(ad609)*
234 *elegans* were injected using standard technique with plasmid *myo-2p*::*mScarlet* in order to label
235 pharyngeal muscle (25 ng/µL). Strong expression of the transgene allows visualization of a single
236 worm when in an aggregate. To perform the assay a standard 5cm NGM plates was seeded with
237 25 µl of *E. coli* OP50 and allowed to dry. To mix the populations, 15 *elegans* expressing *myo-*

238 *2p*::*mScarlet* were placed on the bacterial lawn along with 15 of *monodelphis* or *nigoni*. The plates
239 were placed under a fluorescent dissecting scope and allowed to sit for 45 minutes to mitigate any
240 effects of plate movement. The plate was then imaged using white light (for a reference image)
241 and green light (~546 nm, to capture the *mScarlet* fluorescence). Consistent with previous assays,
242 animals touching two or more individuals were scored as aggregating.
243
244 **Orthology analysis**
245 We used the genome sequence and gene annotations from Wormbase[25] and the *Caenorhabditis*
246 Genome Project to find orthologous gene sets (orthogroups) in non-*elegans Caenorhabditis*
247 species. First, we extracted the longest isoform from every gene using AGAT[26]. Then, using the
248 protein sequence, we constructed orthogroups using OrthoFinder[27,28] using the sequence aligner,
249 Diamond, in ultra-sensitive mode[29].
250
251 **Statistics and reproducibility:**
252 The number for each experiment was based on previous studies and effect size, with each
253 experiment performed with at least 3 independent replicates and each trial performed with matched
254 controls. All data were analyzed and plotted in GraphPad Prism 10 and statistical significance was
255 determined using one-way ANOVA with Tukey's post-hoc test. For comparisons of two data sets,
256 a two-tailed unpaired t-test was used to compare significance. Error bars on figures represent
257 standard error of the mean (SEM) and p-values are shown in each figure to indicate significance
258 ($P<0.05$).
259
260 **<u>Results</u>**
261 **Social feeding behaviors differ across the *Caenorhabditis* clade**
262 We assayed aggregation and bordering behavior using a quantitative imaging setup[16].
263 Assays were performed as previously described, with 50 female animals being used instead of 50
264 hermaphrodites in species that are gonochoristic. In our assay we included four *elegans* control
265 strains; N2 'Bristol' that are solitary, *npr-1(ad609)* that are social feeding, and *npr-1(ad609);nrx-*
266 *1(wy778)* and CB4856 'Hawaiian' *elegans* that both display intermediate social feeding. To
267 observe any possible trends due to species relatedness, the species were ordered based on their
268 phylogenetic relationship and position. We observed wide variation in mean aggregation behavior

269 amongst members of the genus, with some species like *imperialis* and *virilis* showing relatively
270 high levels of aggregation, although not to the level of the *elegans* social control (*elegans npr-*
271 *1(ad609)*)(**Figure 2A-B**). Interestingly, *monodelphis* displays similar aggregation as the *elegans*
272 solitary controls (N2) with little to no aggregation (**Figure 2A-B**). Beyond these three examples,
273 we find that the majority of the species have an intermediate level of aggregation, consistent with
274 a modulated aggregation response. Further, we observe a large level of variation between
275 replicates/experiments within some individual species,, namely *japonica, uteleia, and remanei*
276 (**Figure 2A-B**). Somewhat surprisingly, there was no clear pattern between evolutionary
277 relatedness and the observed level of aggregation. In addition to aggregating during social feeding
278 behavior, the *elegans* aggregates tend to be near or in the border of the bacterial lawn where the
279 bacteria is the thickest (**Figure 2A**)[13]. When we examined bordering behavior across the species,
280 we noted two distinct trends. First, a general downward trend in bordering tendency the more
281 distantly related the species are to *elegans*, and second, that the overall trend of widespread
282 variation within some species is also observed for the bordering behavior (**Figure 2A&C**).

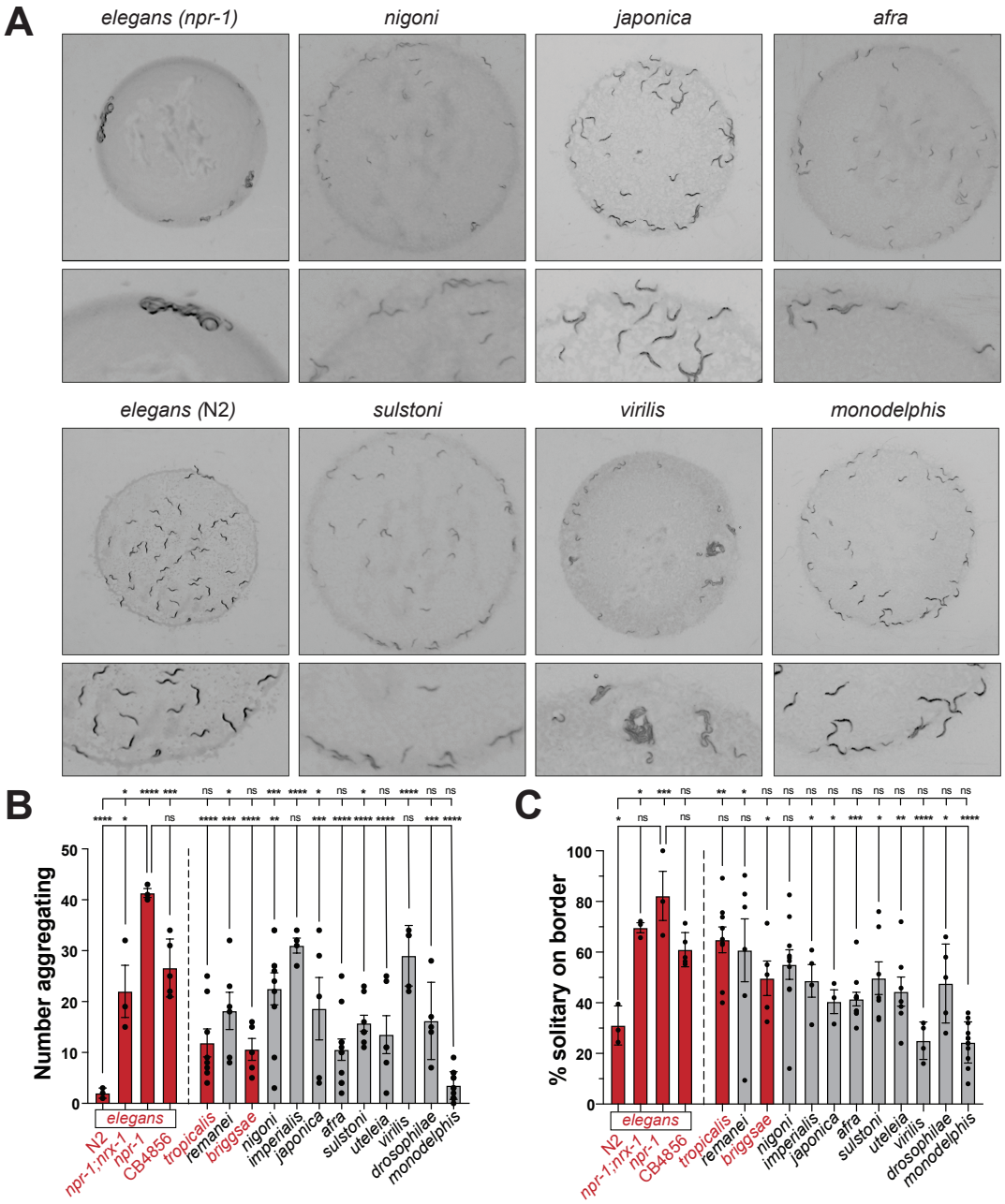


Figure 2. Aggregation behavior is diverse across *Caenorhabditis* clade. (A) Representative images of select *Caenorhabditis* species aggregation feeding and bordering behaviors. **(B)** Quantification of number of aggregating animals in 13 members of the *Caenorhabditis* clade. Species are ordered based on the provided phylogeny (and for subsequent figures). Significant variation is present among members of the clade, and also within individual species. The 4 *elegans* groups assayed represent the negative control (N2 'Bristol'), positive control *npr-1(ad609)*, modulated positive control *npr-1(ad609)*;*nrx-1*(*wy778*), and wild isolate CB4856 'Hawaiian', respectively. Bar graphs represent the mean values across biological replicates (each replicate indicated by a dot). Error bars show stand error of mean. One-way ANOVA with Tukey's post-hoc test was used for statistical comparisons, with p-values indicated in standard format (p=0.05(*), p=0.01(**), p=0.001(***), p= <0.001 (****)). Hermaphroditic species are labeled in

red, while gonochoristic species are labeled in black. **(C)** Quantification of percent solitary animals on food border in 13 members of *Caenorhabditis* clade. Significant variation is present among members of the clade, and also within individual species. Graphical representations and statistical analysis are consistent with previous panel.

To visualize variation among species in both aggregation and bordering phenotypes we generated heat-maps showing the adjusted p-values generated by one-way ANOVA of all combinatorial comparisons (**Figure 3A & B**, respectively). Plotting the phenotypes in a XY plot reveals a relatively broad dispersal, without a clear clustering among closely related species (**Figure 3C**). Overall, the plot emphasizes that while *elegans* N2 and *npr-1*, and *monodelphis* are relative extremes compared to the rest of the species, such extremes occur both in 'domesticated' lab and "wild" populations. Consistent with their independent evolution of hermaphroditism[32], there does not seem to be any significant clustering of the hermaphroditic species. Overall, in characterizing two social feeding behaviors we identified unanticipated levels of variation both among and within species, suggesting our assays may be subject to one or more additional variables that are contributing to variation. Given the wide range of environments from which these species were isolated, we speculated that the lab conditions may represent less than optimal conditions for several species and therefore introduce additional variation.

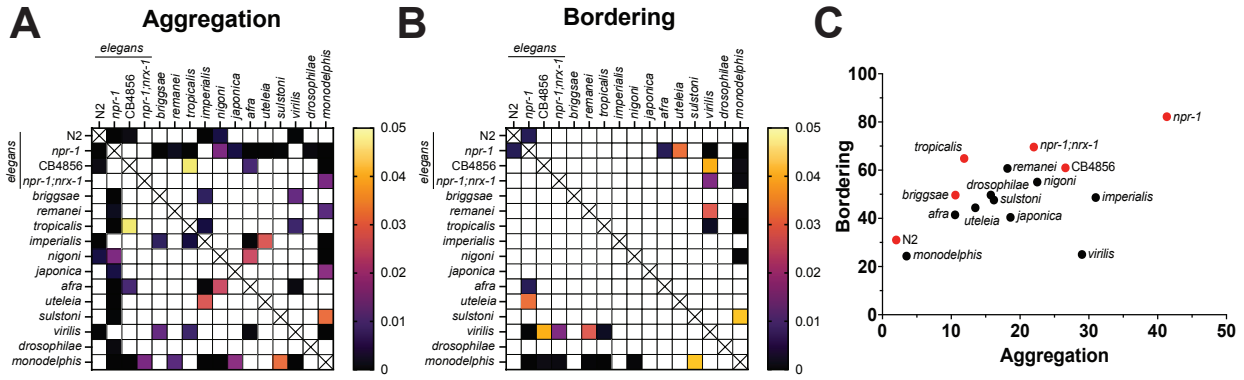


Figure 3. Statistical analysis of phenotypes. (A) Heat-map of ANOVA-generated p-values of all pairwise comparisons in aggregation assay. **(B)** Heat-map of ANOVA-generated p-values of all pairwise comparisons in bordering assay. **(C)** XY data plot showing clustering off species when comparing aggregation and bordering phenotypes. As anticipated, *elegans* N2 and *monodelphis*, and *elegans npr-1* represent the low and high extremes of the cluster, respectively. Consistent with the figures above, hermaphroditic species are labeled in red.

323 **Ecological drivers modulate social feeding behaviors in a species-specific manner**

324 Given the differences in environment and reproductive strategies amongst the species, we
325 reasoned that the makeup of the individual populations could influence social feeding behaviors.
326 To test this hypothesis, we repeated the standard aggregation assays using mixed sex populations
327 (25 males + 25 females, or 25 hermaphrodites in hermaphroditic species with presence of males)
328 (**Figure 4A & B**). For this assay we focused on a subset of the species displaying high level of
329 variation in the female/hermaphrodite only assays, in part to test if a more "ecologically relevant"
330 populations might reduce some of the observed variation. Overall, for most species the mixed sex
331 populations did not alter aggregation behavior compared to the single sex populations (**Figure 4A**
332 **& B**). However, *monodelphis* and *tropicalis,* showed significantly increased aggregation in the
333 mixed sex population assay compared to the single sex hermaphrodite/female population assay
334 (**Figure 4A**). These results again highlight the impact of local environment and context on
335 variation in behaviors between species, whereas these assays reflect a modulated response to real-
336 time sensory-environment landscape.

337 To explore other ecologically relevant population dynamics (such as interspecies
338 cohabitation and interactions, for example escape from predation[33]), we tested whether combining
339 additional species with *elegans* would influence *elegans* social feeding behavior. We first tested
340 whether a reduced population size (30 animals) or the addition of a *myo-2p* fluorescent transgene
341 would impact the aggregation behaviors of each species. Aggregation in both *nigoni* and
342 *monodelphis* were significantly reduced compared to *elegans* (15 *myo-2p* positive(+) and 15 *myo-*
343 *2p* negative(-))(**Figure 4C**) and significantly different from one another, which is consistent with
344 the 50 animal aggregation assay results (**Figure 2**). Furthermore, the inclusion of the *myo-2p*
345 transgene did not impact *elegans npr-1*(*ad609*) aggregation behavior. Next, we mixed 15 *myo-2p+*
346 *elegans npr-1(ad609)* social feeding controls with 15 *myo-2p- elegans, monodelphis,* or *nigoni* on
347 a seeded NGM plate and allowed the mixed populations to acclimate for 45 minutes. After the
348 acclimation period, the plates were imaged and assayed for aggregation behavior. We aimed to
349 understand if the mixing of two distinct populations would alter the aggregate feeding dynamics
350 of either or both species. In the mixed species population assays, we noted that addition of *nigoni*
351 or *monodelphis* to the *myo-2p+ elegans* population significantly reduced aggregation behavior of
352 *elegans* (**Figure 4D&E**). Interestingly, we did not observe an impact of *elegans* on behavior of
353 the other species. We reasoned that disruption of *elegans* aggregation behavior could be the result

of a number of changes to environmental cues present on the plates that result from mixing populations.

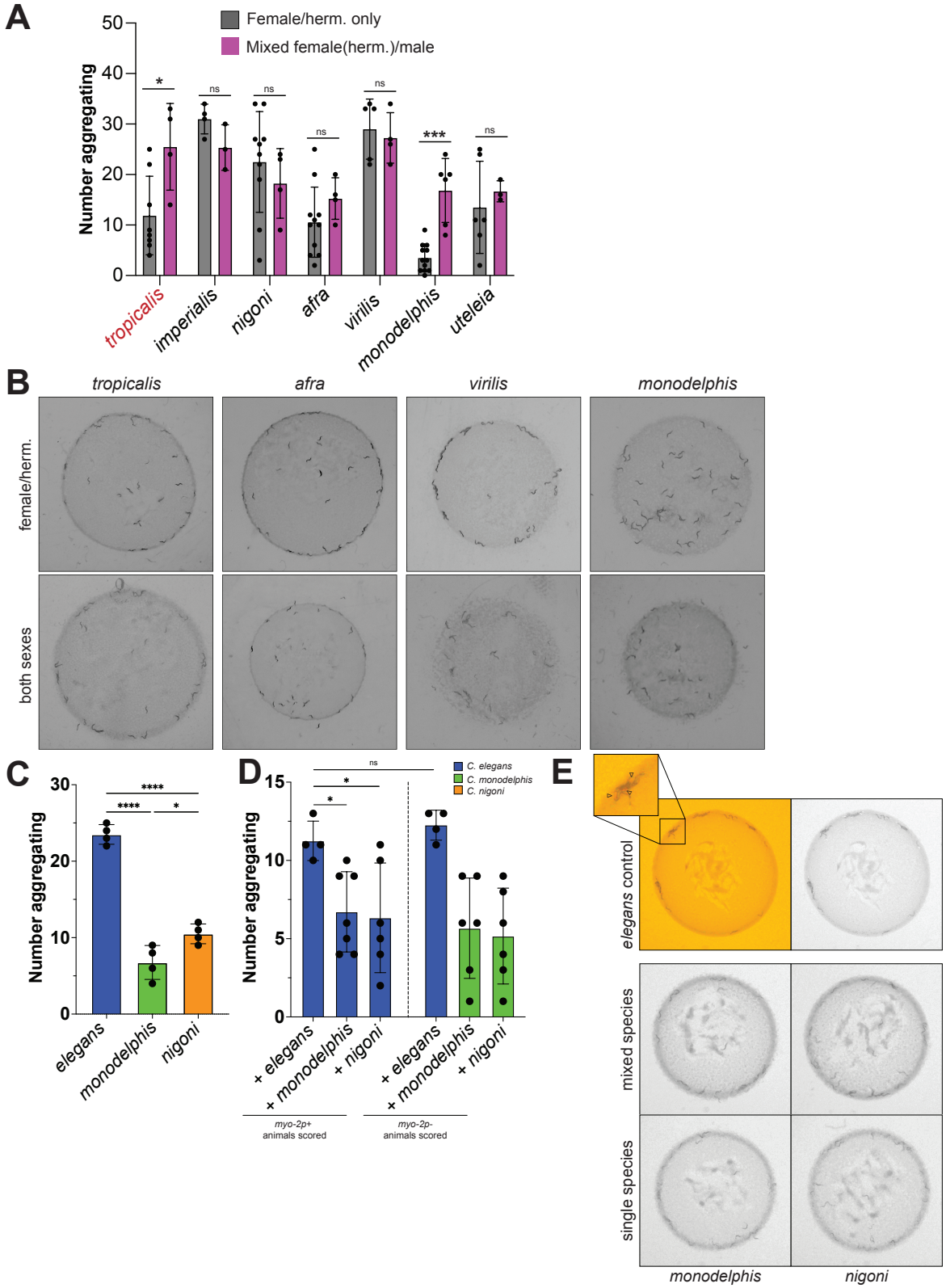


Figure 4. Ecological drivers modulate social feeding behaviors in a species-specific manner. **(A)** Representative images and **(B)** quantification of number of aggregating animals of *Caenorhabditis* species in mixed sex populations. *tropicalis* and *monodelphis* both showed significant differences in aggregation when males were added to the population. Bar graphs represent the mean values across biological replicates (each replicate indicated by a dot). Error bars show stand error of mean. Standard T-test was used for individual comparisons; p-values are indicated in standard format (p=0.05(*), p=0.01(**), p=0.001(***), p= <0.001 (****)). Hermaphroditic species are labeled in red, while gonochoristic species are labeled in black. **(C)** Quantification of 30 animal single-species controls. **D)** Quantification of mixed species assays, where 15 *elegans myo-2p*+ animals were mixed with 15 *elegans myo-2p-, monodelphis,* or *nigoni*. Combining *elegans* and *nigoni* significantly lowers aggregation of *myo-2* expressing *elegans* compared to their *elegans* alone control (left), while addition of monodelphis caused an insignificant but trending reduction (left). *monodelphis* and *nigoni* have significantly lower aggregation than their *elegans myo-2p*– control (right). **(E)** Representative images of mixed species assays. *myo-2p* expressing *elegans* are indicated by arrows (top left window).

Caenorhabditis **clade has high level of conservation in NPR-1 receptor protein**

Given the variation in the social feeding behaviors in the *Caenorhabditis* genus, we began to consider what mechanisms might drive species differences. Morphological differences likely drive some of the changes, however, there has been very little characterization in most of the species we assayed. There are several papers examining the development of the vulva [34–36], tail morphology[37–39] and body size[40], however there is still a significant deficit in the literature. This lack of species characterization is especially apparent when considering the nematode nervous systems, with the only other *Caenorhabditis* species with any significant neuronal characterization being *briggsae*[41,42]. To bypass the lack of circuit-based knowledge within the clade, we leveraged several known genetic drivers of social feeding behavior in *elegans*, notably the neuropeptide receptor NPR-1. To compare *npr-1* across species, we first queried both existing and draft genome assemblies for orthologues using OrthoFinder[28]. Given the size and relative domain conservation of the NPR family of genes in *elegans* we were somewhat surprised to identify a single orthologue of NPR-1 in all species except for *nigoni*, which had multiple related paralogues and was thus excluded from our analysis. To characterize sequence conservation among species we used CLUSTAL V5.0 analysis to perform a multiple sequence alignment to produce an NPR-1 alignment (**Figure 5A**). The alignment showed there is a high level of conservation among the species, even when considering the most evolutionary divergent species, like *monodelphis*. We queried three residues in NPR-1 that when mutated have been shown to induce social behaviors[13] and found them to be well conserved and wildtype.

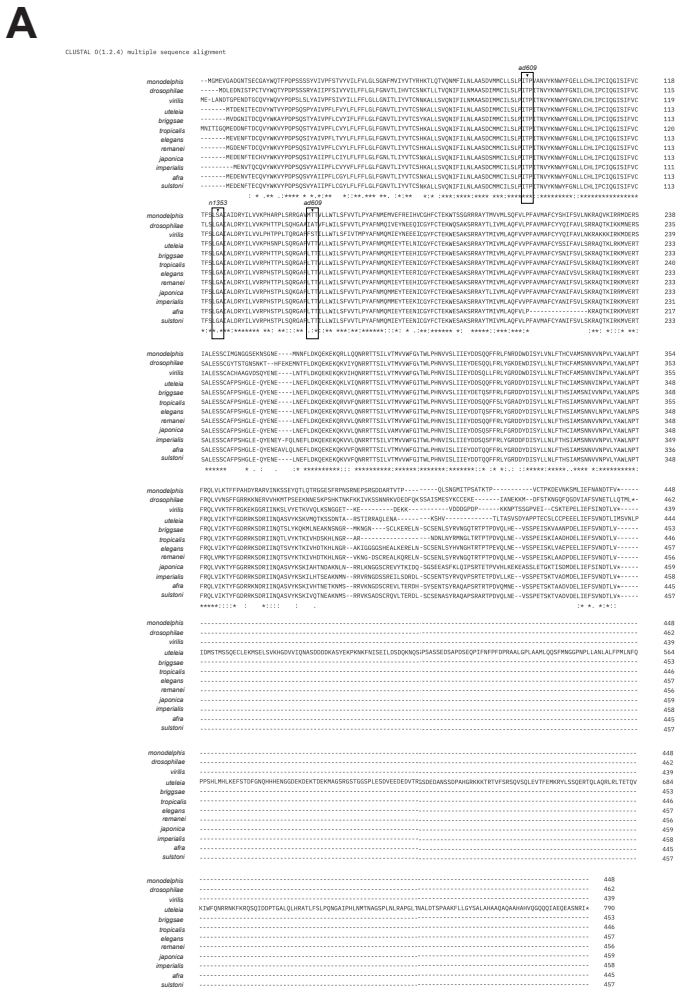


Figure 5. Protein alignment of NPR-1 orthologues in amongst *Caenorhabditis* nematode species. **(A)** NPR-1 orthologues were queried from existing *Caenorhabditis* genomes or assemblies based on sequence similarity. A single orthologue was identified in each species with the exception of *nigoni* which was excluded due to the lack of a confident orthologue. Overall, NPR-1 is relatively well conserved across the clade.

Discussion

Here, we examined several social foraging behaviors of an evolutionarily diverse clade of *Caenorhabditis* nematodes to better understand how such behaviors differ and may have evolved between species. Across these divergent species of nematodes, we noted several surprising trends within a set of social feeding behaviors that underpin a shared general feeding strategy. Notably, we confirmed *elegans* (*npr-1*) is a relative extreme when compared to several wild isolates and other species. In contrast, most of the species we assayed (including the wild *elegans* isolate CB4856 'Hawaiian' strain) showed intermediate levels of aggregation, while basal taxon

408 *monodelphis* showing little to no aggregation, not dissimilar to *elegans* N2, which is known to be
409 solitary due to a gain of function mutation in the *npr-1* gene. Furthermore, we uncovered a
410 surprising trend when examining bordering behavior among species, notably that the phenotype
411 seems to generally decline the further away the species is in relatedness to *elegans*. While this
412 trend is not perfect, it does again highlight the idea that N2 'Bristol' *elegans* may represent a
413 behavioral outlier when compared with wild *elegans* isolates and most other species.

414 We noted that moderate to high levels of variation were present in most species when
415 considering both aggregation and bordering phenotypes. The extensive variation present within
416 several of the species raises the possibility that an active mechanism is driving heterogeneity
417 beyond the expected variation present in most behaviors. This unexpected variation could be in
418 part due to genetic heterogeneity present between individuals, maintained in part through selection
419 pressure. *elegans* strains have been artificially selected and lab-evolved (in part because they
420 reproduce clonally), while in comparison, most of the species we assayed represent far more
421 heterogeneous populations. Several species have been backcrossed and inbred extensively from
422 their wild isolates, effectively rendering them lab strains **(Table 1)** after undergoing selection for
423 domestication. We can speculate that some of the species likely retain higher levels of genetic
424 heterogeneity, especially among genetic elements such as single nucleotide variations (SNPs).
425 While the vast majority of SNPs are unlikely to be the causative agent directly underlying a
426 phenotypic change, single nucleotide changes can drastically alter behavior, as highlighted by
427 single nucleotide variation in the neuropeptide receptor NPR-1. Many wild isolates of *elegans*
428 naturally aggregate and display social feeding, but in the lab N2 Bristol strain, a single adenine to
429 guanine substitution in NPR-1 is sufficient to produce a gain of function phenotype, eliminating
430 social behavior and inducing solitary feeding[13]. Lastly, it is not unreasonable to speculate that
431 genetic variants present within individuals may have a modulatory effect within particular
432 behavioral circuits, especially if they are closely associated with critical neuronal genes or
433 regulatory elements[43]. This concept is highlighted in recent work showing mutations in the *daf-19*
434 transcription factor are able to dramatically alter chemosensory and oxygen-sensing circuits
435 leading to a disruption of social feeding behaviors in both *C. elegans* and *P. pacificius*[44].

436 Outside of genetic mechanisms, it is very likely that some of the variation seen in social
437 feeding behaviors is a result of the lab environment in which these assays are being performed. In
438 the lab we make every effort to maintain temperature at 20 °C with low to moderate humidity,

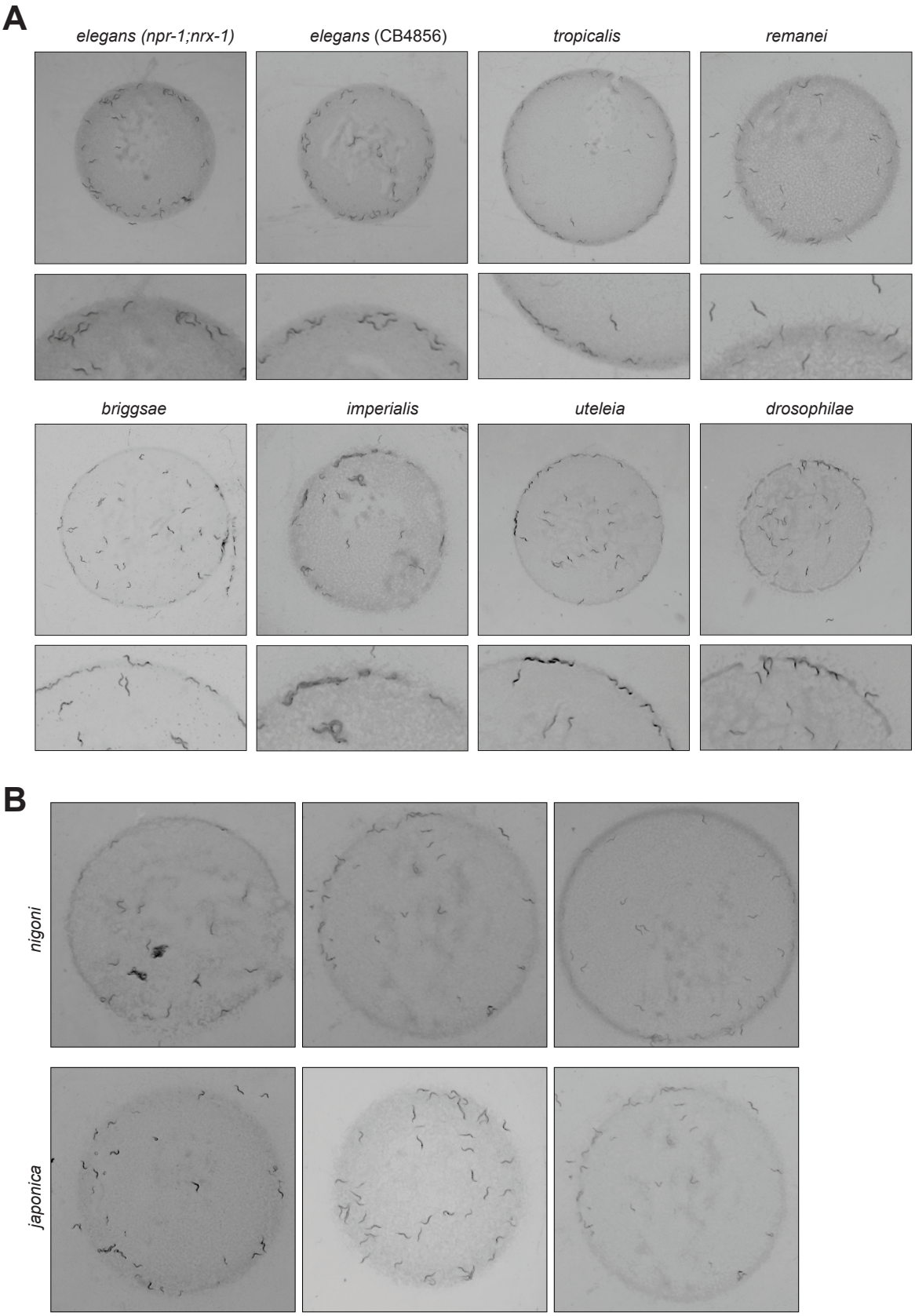
439 which is ideal for the growth and maintenance of lab strains like *elegans* and *briggsae*. However,
440 many of the other *Caenorhabditis* species were isolated from drastically different climates and
441 environments and likely underwent adaptation of their environmental-sensory circuits to these
442 divergent conditions in contrast to those in which we assayed them. While testing ecologically
443 relevant temperatures and humidity levels for each species is logistically beyond the scope of this
444 study, one might imagine that social feeding behaviors might be altered if those conditions more
445 accurately reflect what occurs in the wild.

446 Furthermore, the substrate on which we feed the animals is resoundingly artificial, using a
447 monoculture of a lab strain of *E. coli* (OP50) on an agarose surface is hardly representative of the
448 natural ecology of wild feeding substrates. When considering the natural environments in which
449 these nematodes normally reside, the microbiomes can also be dramatically heterogenous
450 depending on location. Most natural feeding substrates contain a range of bacterial species, some
451 of which are viable food sources and some that may represent nematode pathogens. To reconcile
452 these differences, recent research has characterized food choice preferences in *elegans*, uncovering
453 their preference for *bacillus* species over the traditional lab *E. coli* strains[45]. Even within *E. coli*
454 species, *elegans* tend to prefer nutrient dense strains, likely to supplement for essential nutrients,
455 and potentially leading to changes in metabolism[45,46]. It is not surprising then that different food
456 sources can lead to dramatic variations in behavior. Best studied is the attraction/avoidance
457 response to food, which allows the animals to avoid potentially pathogenic bacteria while
458 optimizing nutrient uptake[47,48]. Lastly, *elegans* has been shown to modulate their feeding behaviors
459 based on the density of food, where both solitary and social strains disperse when food density is
460 strongly diminished[14], likely as part of a co-dependent circuit detecting both starvation cues and
461 increased oxygen[49].

462 The last major factor potentially influencing these behaviors is chemical signaling in the
463 form of environmental secretions and touch stimuli. Although the nature of these chemical
464 modulators is widely unknown, they represent an intriguing mechanism by which individuals can
465 modulate interactions with other animals and their environment. For example, it is well known that
466 several species of nematodes have complex and diverse secretomes, which they use to modulate
467 interactions with their environment. Several studies have shown that parasitic nematodes secrete
468 protein or chemical modulators to downregulate their host's immune response[50]. Similarly, the
469 *elegans* genome encodes a large number of putative secreted proteins[51], some of which were

470 isolated via mass spectrometry, identifying a number of novel and nematode specific protein
471 factors of unknown function. In parallel, the chemical secretome of *elegans* revealed a complex
472 biochemical landscape, heavily enriched in putative pheromones, especially ascarosides[52]. Given
473 the unknown function of many of these proteins and chemicals it is not unreasonable to assume at
474 least a subset may be influencing the behaviors of both individuals and populations of individuals.
475 Several ascaroside pheromones for example are well documented to alter population behaviors,
476 modulating hermaphrodite-male attractions and repulsion, mating, and dauer formation in the
477 absence of food[53]. Furthermore, pheromone secretion detected in the environment can also induce
478 the production of EV's in neuronal tissues of the affected animals, potentially altering behavior[54].
479 Taken together we must consider that unknown signaling molecules are modulating the interaction
480 between individuals in our social feeding assays. Among the individuals of the same species, these
481 signals may provide a real-time readout of the local sensory environment helping animals to
482 respond and alter their behavior accordingly. Among individuals of different species, the
483 interactions are potentially even more complex. Differences in pheromone usage or chemical
484 secretome might produce novel interactions leading to interesting behavioral variations.

485 Overall, this work represents the first broad characterization of social feeding behaviors
486 across a large and evolutionarily ancient lineage of nematodes. Given the range of distinct
487 environmental conditions in which these species naturally reside, it may not be surprising that we
488 observed variation among species across several behavioral paradigms. The current circuit
489 mechanism underlying the social feeding behaviors is still trying to fully reconcile how behavior
490 is tuned in response to various sensory cues like food identity/density[14,45,46] and O_2/CO_2 balance[55].
491 Complicating matters is recent evidence in *P. pacificus* showing that single cues such as elevation
492 (and corresponding depletion of O_2) can override the established behavioral paradigm and
493 introduce variation[15]. Context-dependent variation is likely to complicate dissection of the genetic
494 and molecular mechanisms of behavioral variation, but presents an exciting opportunity to study
495 real-time plasticity of behaviors and neuronal circuits.


A
elegans (npr-1;nrx-1)
elegans (CB4856)
tropicalis
remanei
briggsae
imperialis
uteleia
drosophilae
B
nigoni
japonica

496

Supplementary Figure 1. Aggregation behavior is diverse across *Caenorhabditis* clade. (A) Representative images of additional species in the *Caenorhabditis* clade. **(B)** Multiple representative images of *nigoni* and *japonica* showing variation in aggregation and bordering behaviors.

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Author Contributions

DH and MPH conceived and designed the study and experiments, and DH, JP, and RFHE conducted all experiments. DH processed, analyzed, and interpreted all data with help from CRLL. DH wrote the manuscript with assistance from MPH, and all authors reviewed, revised, and approved the manuscript.

Competing Interests

The authors declare no conflicts of interest.

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