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

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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 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 wide variation in aggregate social feeding and bordering behaviors of hermaphrodite and female animals, but the variation did not correlate with evolutionary relatedness of the species. Combination of both sexes of individual species increased aggregation behavior of select species that had lower levels of aggregation in single sex assays. Addition a second species with *C. elegans* significantly reduced aggregate feeding behavior of *C. elegans*, but not the other species. Intraspecies and interspecies interactions can modify 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 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 not only in the
92 complexity of their specialization (including the evolution of differentiated and highly specialized
93 neuronal tissues) but also in the manner and breadth of their behavioral responses to environmental
94 stimuli [2,3]. The evolution of complex behavior can be loosely correlated with the evolution of
95 multicellularity and complex tissues[4]. Likewise, tissue differentiation also gave rise to neural
96 tissues, which in their simplest form, link networks of sensory cells together forming a basic neural
97 circuit. In more complex Eukaryotes, more sophisticated neural networks, utilizing multiple
98 sensory and neuronal cell types form the basis of complex organs such as neural ganglion, nerve
99 tracts, and the brain [5,6]

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

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

116 Amongst common lab models, the nematode *Caenorhabditis elegans* is a particularly
117 powerful tool for studying behavior. In addition to the many genetic tools, a fully mapped neural

118 connectome (chemical, electrical, and peptidergic) provide a powerful model to link stereotyped
119 behaviors with alterations of neuronal activity at both a single cell and circuit level. In addition,
120 the *Caenorhabditis* clade of nematodes represents a unique opportunity to study evolution of
121 behavior in a diverse and evolutionarily ancient lineage **(Figure 1)**. Nematodes of this clade are
122 found in a worldwide dispersal, with populations found in nearly every ecological niche probed
123 for their presence[10]. In the wild, members of the *Caenorhabditis* nematodes persist in a free-living
124 state generally colonizing bacteria-rich substrates, such as rotting fruit, allowing them to utilize
125 the local microbiota as food source. Considering their global dispersion, individual members of
126 the clade experience significant variation in local environment, including differences in
127 temperature, humidity, concentration of oxygen, and dominant microbiota[11]. Notably, these
128 species are rarely isolated from homogenous populations; *elegans* for example is frequently co-
129 isolated with *briggsae* and/or *remanei* from the same substate. Altogether, the *Caenorhabditis*
130 clade presents a compelling model through which to query the evolution of behavior across a large
131 evolutionary timescale and a wide range of 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 shared
134 feeding strategies. One of the most well-characterized feeding behaviors is termed social feeding
135 and is defined by social interactions that occur between individuals feeding in the same local
136 environment[12]. Although not well defined, one can speculate that this behavior has costs and
137 benefits for individual animals. Animals may be attracted to high density food that is resource rich
138 for obvious nutrient seeking reasons, but animals populating a food/bacteria rich location can also
139 result in spreading of the food source (bacteria) to surrounding areas. Further, group feeding could
140 facilitate short-distance signaling between animals (perhaps through pheromones) and/or increase
141 opportunities for mating, but it seems clear that this behavior is environment and context
142 dependent[13,14]. Early surveys of wild *elegans* isolates showed a surprisingly high level of
143 variability in social feeding behaviors. Contrary to the widely used N2 Bristol lab strain, which
144 displays solitary feeding, many wild isolates from a range of environments show higher levels of
145 social feeding[12]. de Bono & Bargmann attributed the solitary feeding behavior of N2 Bristol strain
146 to a gain of function missense mutation in the *npr-1* gene (ortholog of neuropeptide Y receptor).
147 This mutation results in an amino acid change in the third intracellular loop of the NPR-1 G-protein
148 coupled receptor , whereas loss of function mutants in the *npr-1* gene result in social feeding in

149 *elegans*[12]. For the purpose of this study we utilize the *npr-1(ad609)* loss of function mutant to
150 induce social feeding in the N2 lab control strain, which represents the most reliable and robust
151 positive control for social feeding behaviors[15].

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 [13,16–19]. 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[16,17]. Genetic dissection of this sensory circuit
158 has uncovered distinct roles for the neuropeptide receptor NPR-1[12,15] and gap junction connections
159 between involved sensory neurons and the RMG interneuron[16,20]. 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[15]. Therefore, social feeding behavior relies on co-
163 dependent signaling of neuropeptides, and electrical and chemical synapses. Together, these
164 mechanisms serve to fine-tune the balance between solitary and social feeding behaviors[15]. Given
165 the complexity of this behavioral circuit, we set out to test whether social feeding behavior is
166 conserved throughout the *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[21]. Two large
171 subgroups, *elegans* and *japonica*, have emerged based on genomic data providing multiple
172 lineages to interrogate. *monodelphis*, representing the basal taxon of the clade, is estimated to have
173 diverged over 100 million years ago, an evolutionary timescale far beyond that of the split between
174 mice and humans [22]. Lastly, the true strength of this model lies the opportunity to study "wild"
175 populations of nematodes (representing both hermaphroditic and gonochoristic species) in a
176 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 from which future work investigating differences in sensory cues,
179 neuronal function, and circuitry can be launched.

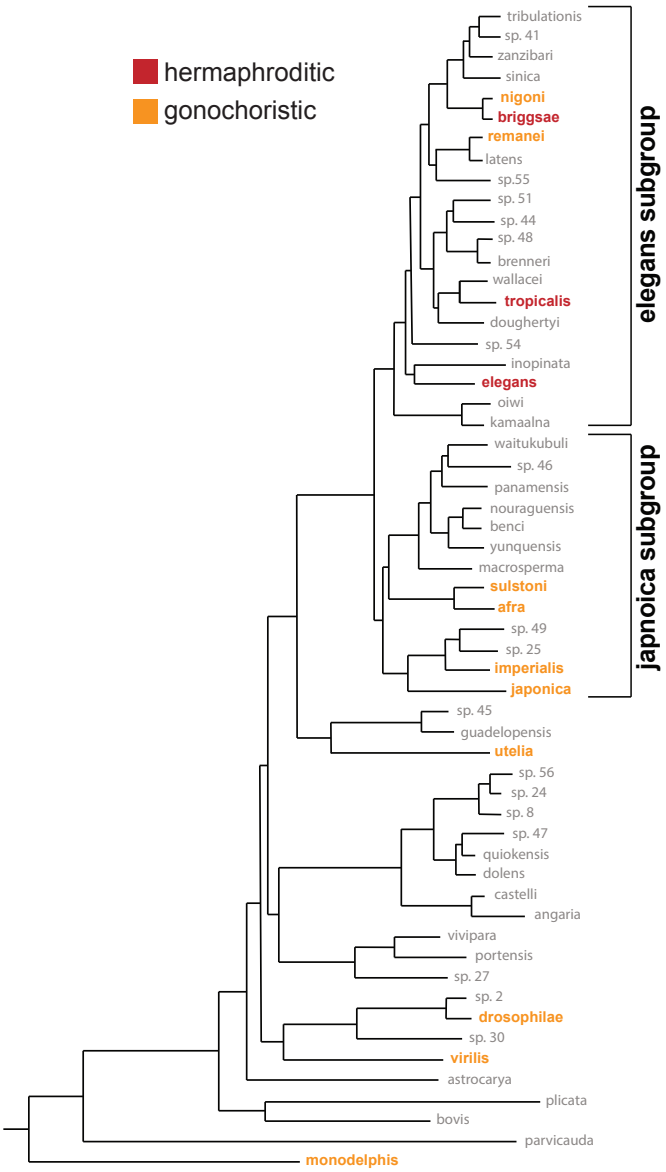


Figure 1. The *Caenorhabditis* clade of nematodes is an evolutionary and diverse lineage. (A) Phylogenetic tree of *Caenorhabditis* clade showing the two large subgroups (*elegans* and *japonica*) and the basal taxon *monodelphis* (adapted from *Caenorhabditis* Genome Project). Species included in behavioral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

Using the twelve members of the *Caenorhabditis* clade we have 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 display significantly more social feeding that the *elegans* solitary control strain. Furthermore, by testing more ecological relevant populations in our assays

192 we have begun to tease out the impact of intraspecies and interspecies interactions. Mixing males
193 with females of individual species altered aggregate feeding behavior in two species when
194 compared to homogeneous female populations. Lastly, combining a second species with social
195 feeding *elegans* was sufficient to significantly reduce aggregation feeding of *elegans*. This work
196 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 **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

200

201 **<u>Methods</u>**

202 **Strains Maintenance**

203 Animals were maintained under normal growth conditions (~20° C) on normal NGM media[23].
204 Plates were seeded with *E. coli* OP50 to provide food. Strains utilized in this study are listed in
205 **Table 1**. *npr-1*(*ad609*) and *nrx-1*(*wy778*) mutations were confirmed in *elegans* strains and were
206 used as positive controls. All other species were obtained from the CGC and maintained by
207 chunking.
208
209 **Social feeding behavior assay**
210 Standard 6-well culture plates were filled with 6 mLs of standard NGM media and then seeded
211 with 75 μL of *E. coli* OP50 and allowed to dry overnight. For the standard assay, 50 animals were
212 picked onto clean plates and then moved onto the seeded wells, transferring as little bacteria as
213 possible to prevent buildup. For standard social feeding and bordering assays (**Figure 2B&C**) the
214 50 animals were either female or hermaphrodites depending on the species. For the mixed sex
215 assays, we combined 25 males with 25 females/hermaphrodites (depending on species).
216 Regardless of the composition of the assay, worms were picked during and placed on the assay
217 during their fourth larval stage. Female or hermaphrodite animals were staged based on the
218 presence of half-moon shape characteristic of a L4 developing vulva. Males were staged based on
219 relative size, germline features, and morphological development of the tail. After placing the
220 animals on the 6-well plate, a small amount of Tween20 was used to thinly coat the lid to prevent
221 condensation forming during imaging. The plates are imaged using the WormWatcher automated
222 imaging platform (Tau Scientific) for a total of 20 hours with a cluster of 10 images (over 1 minute
223 timecourse) being taken every hour. To quantify social feeding behavior each well was manually
224 scored from blinded images, where a worm was considered aggregating if was contacting two or
225 more other worms[12,15]. Bordering was assayed by calculating the percentage of solitary animals
226 that we in direct contact with the border region of the bacterial lawn (with the border defined as
227 approximately half a body length in width). Data shown is from the 15-hour mark, which placed
228 the animals of all species in the middle of Day 1 of adulthood.
229
230 **Mixed species assays**
231 In order to assay the effect of mixed species, we first generated a fluorescently tagged
232 *elegans* strain to differentiate them from the other species while imaging behavior. *npr-1(ad609)*
233 *elegans* were injected using standard technique with plasmid *myo-2p*::*mScarlet* in order to label

234 pharyngeal muscle (25 ng/µL). Strong expression of the transgene allows visualization of a single
235 worm when in an aggregate. To perform the assay a standard 5cm NGM plates was seeded with
236 25 µl of *E. coli* OP50 and allowed to dry. To mix the populations, 15 *elegans* expressing *myo-*
237 *2p*::*mScarlet* were placed on the bacteria lawn along with 15 of *monodelphis* or *nigoni*,. The plates
238 were placed under a fluorescent dissecting scope and allowed to sit for 45 minutes to mitigate any
239 effects of plate movement. The plate was then imaged using white light (for a reference image)
240 and green light (~546 nm, to capture the *mScarlet* fluorescence). Consistent with previous assays,
241 animals touching two or more individuals were scored as aggregating.
242
243 **Orthology analysis**
244 We used the genome sequence and gene annotations from Wormbase [24] and the *Caenorhabditis*
245 Genome Project to find orthologous gene sets (orthogroups) in non-*elegans Caenorhabditis*
246 species. First, we extracted the longest isoform from every gene using AGAT[25]. Then, using the
247 protein sequence, we constructed orthogroups using OrthoFinder[26,27] using the sequence aligner,
248 Diamond, in ultra-sensitive mode[28].
249
250 **Statistics and reproducibility:**
251 The number for each experiment was based on previous studies and effect size, with each
252 experiment performed with at least 3 independent replicates and each trial performed with matched
253 controls. All data were analyzed and plotted in GraphPad Prism 10 and statistical significance was
254 determined using one-way ANOVA with Tukey's post-hoc test. For comparisons of two data sets,
255 a two-tailed unpaired t-test was used to compare significance. Error bars on figures represent
256 standard error of the mean (SEM) and p-values are shown in each figure to indicate significance
257 ($P<0.05$).
258
259 **Results**
260 **Social feeding behaviors differ across the *Caenorhabditis* clade**
261 We assayed aggregation and bordering behavior using a quantitative imaging setup[15].
262 Assays were performed as previously described, with 50 female animals being used instead of 50
263 hermaphrodites in species that are gonochoristic. In our assay we included four *elegans* control
264 strains; N2 'Bristol' that are solitary, *npr-1(ad609)* that are social feeding, *npr-1(ad609);nrx-*

265 *1(wy778)* that display intermediate social feeding, and CB4856 'Hawaiian' *elegans* that display
266 intermediate social feeding. We observed wide variation in mean aggregation behavior amongst
267 members of the genus, with some species like *imperialis* and *virilis* showing relatively high levels
268 of aggregation, although not to the level of the *elegans* social control (*elegans npr-*
269 *1(ad609)*)(**Figure 2A-B**). Interestingly, *monodelphis* essentially phenocopies the *elegans* solitary
270 controls (N2) with little to no aggregation (**Figure 2A-B**). Outside these three examples, we find
271 that the majority of the species have an intermediate level of aggregation, consistent with a
272 modulated aggregation response. Further, we observe that within some individual species, a large
273 level of variation between replicates/experiments, namely in *japonica, uteleia, and remanei*
274 (**Figure 2A-B**). Somewhat surprisingly, there was no clear pattern between evolutionary
275 relatedness and the observed level of aggregation. In addition to aggregating, when social feeding
276 behavior occurs, *elegans* aggregate in groups near or in the border of the bacterial lawn where the
277 bacteria is the thickest[12] (**Figure 2A**). When we examined bordering behavior we noted two
278 distinct trends. First, a general downward trend in bordering tendency the more distantly related
279 the species are to *elegans*, and second, that the overall trend of widespread variation is also
280 observed when considering this bordering phenotype (**Figure 2A&C**).

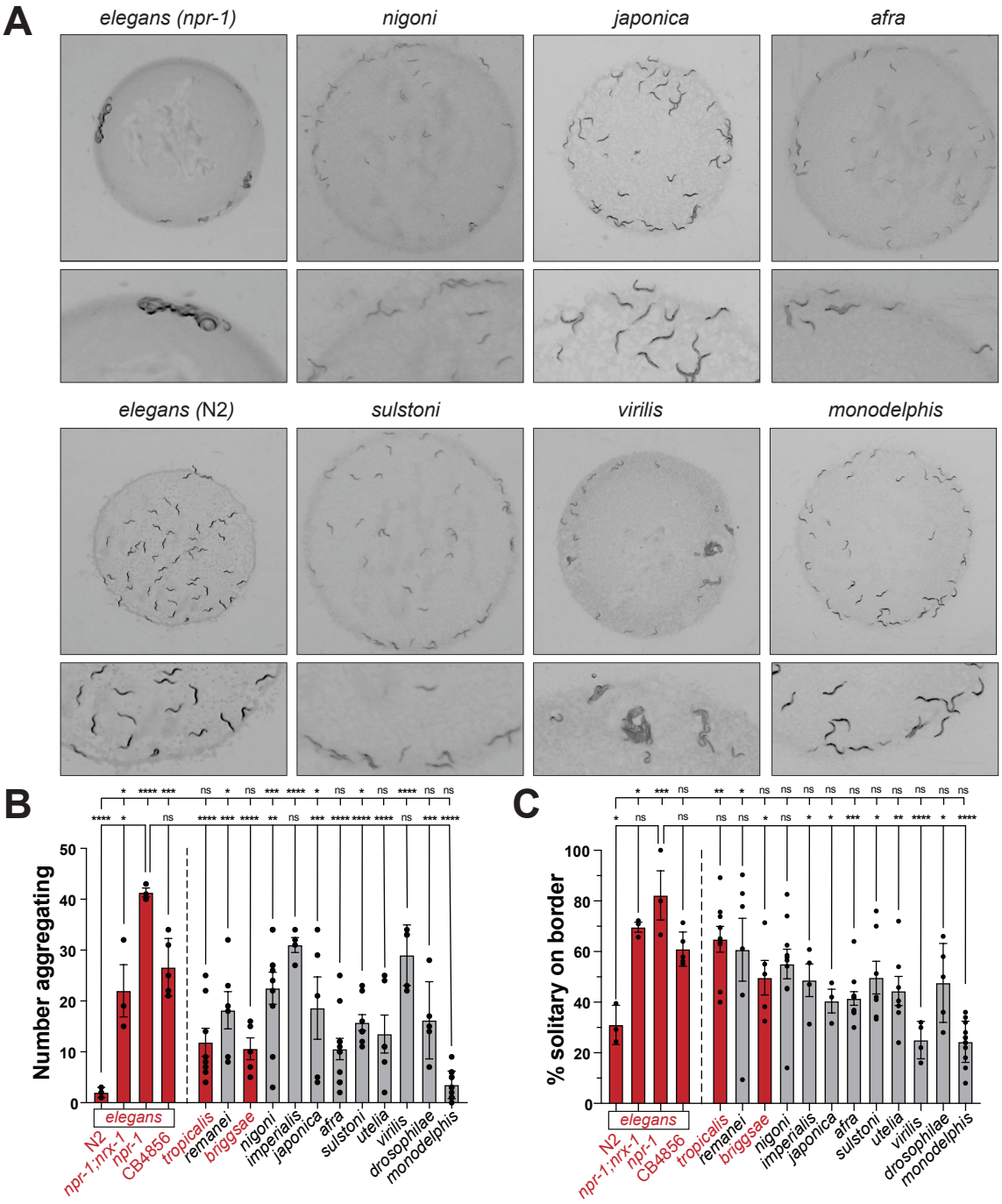


Figure 2. Aggregation behavior is diverse across *Caenorhabditis* clade. (A) Representative images of select *Caenorhabditis* species aggregation feeding and bordering behaviors. **(B)** Quantification of number of aggregating animals in 13 members of the *Caenorhabditis* clade. Significant variation 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 % solitary animals on 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 that *elegans* N2 and *npr-1*, and *monodelphis* are relative extremes compared to the rest of the species, such extreme can occur both in domesticated and "wild" populations. Consistent with their independent evolution of hermaphrodism[31], 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 out assays may be subject to one of 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 an additional element of variation.

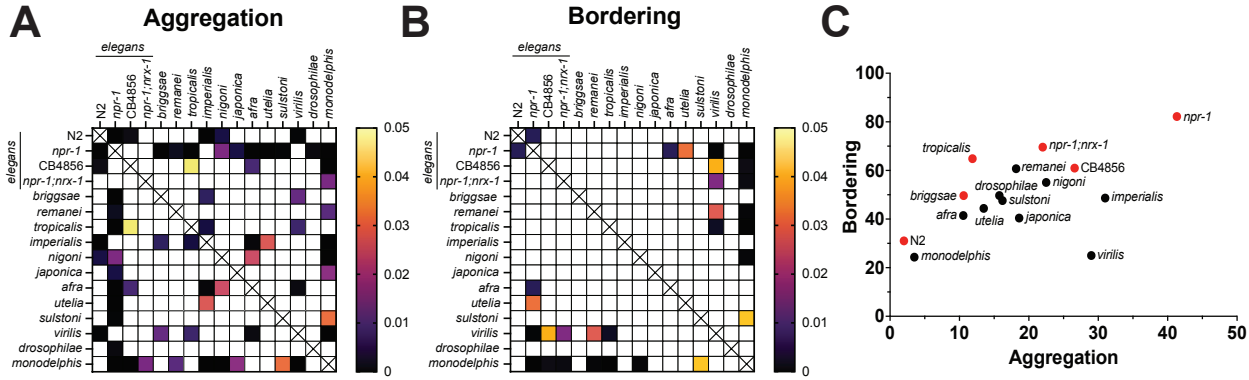
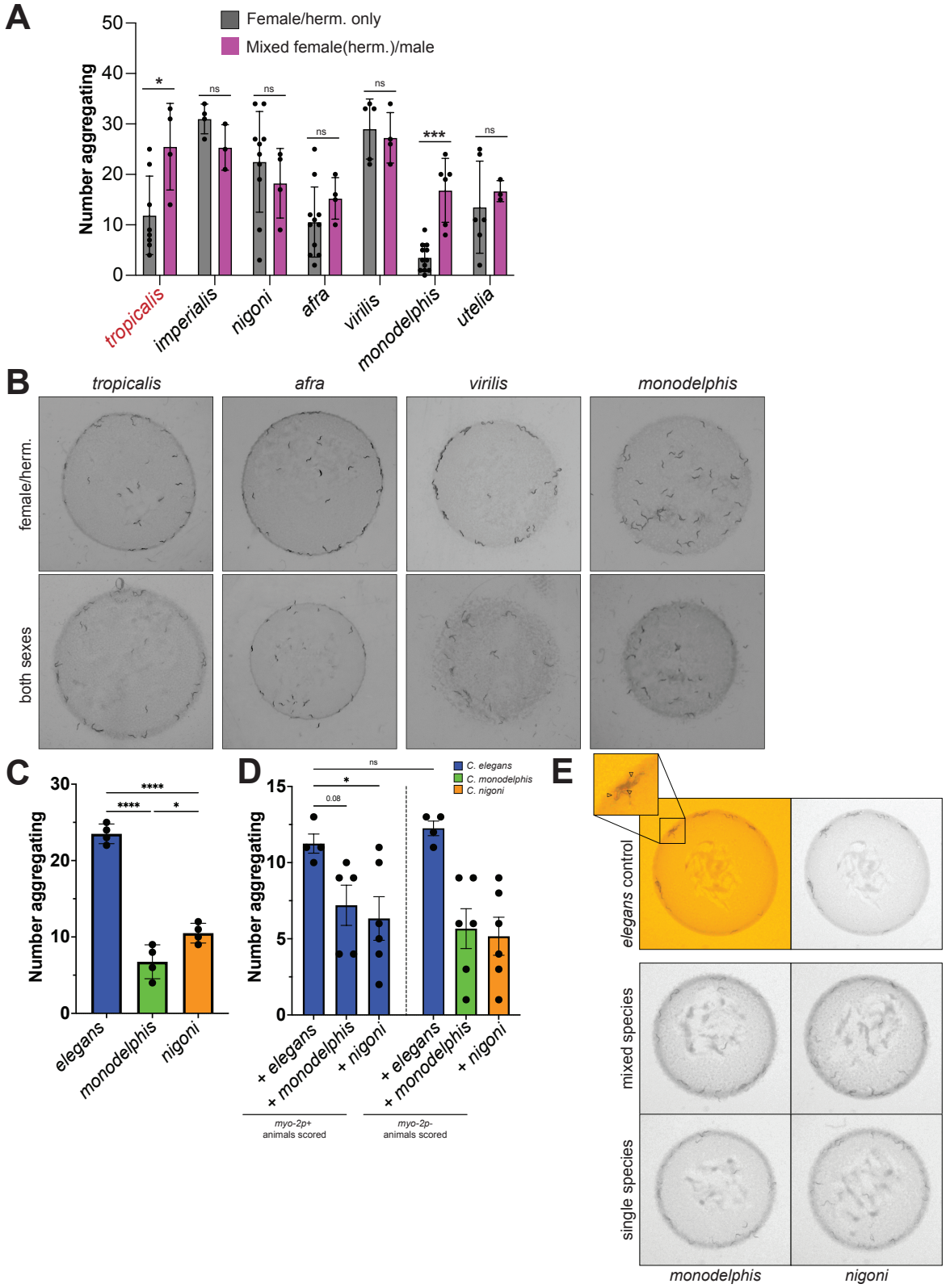


Figure 3. Statistical analysis of phenotypes. (A) Heat-map of ANOVA-generated p-values of all pairwise comparisons in aggregation assay. **(B)** Heat-map of ANOVA-generated p-values of all pairwise comparisons in bordering assay. **(C)** XY data plot showing clustering off species when comparing aggregation 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.

Ecological drivers modulate social feeding behaviors

319 Given the differences in environment and reproductive strategies amongst members of the
320 genus we reasoned that the makeup of the individual populations may influence social feeding
321 behaviors. To test this hypothesis, we repeated the standard aggregation assays using mixed sex
322 populations (25 males + 25 females, or 25 hermaphrodites in hermaphroditic species with presence
323 of males) (**Figure 4A & B**). For this assay we focused on a subset of the species displaying high
324 level of variation in the female/hermaphrodite only assays, in part to test if a more "ecologically
325 relevant" populations might reduce some of the observed variation. Overall, for most species the
326 mixed sex populations did not alter aggregation behavior compared the single sex populations
327 (**Figure 4A & B**). However, *monodelphis* and *tropicalis,* showed significantly higher aggregation
328 in the mixed sex population assay compared to the single sex hermaphrodite/female population
329 assay (**Figure 4A**). These results again highlight the impact of local environment and context on
330 variation in behaviors, whereas these assays reflect a modulated response to real-time sensory-
331 environment landscape.
332 To further explore other ecologically relevant population dynamics (such as interspecies
333 cohabitation and interactions, for example escape from predation[32]), we tested whether combining
334 additional species with *elegans* would influence their social feeding behavior. We first tested
335 whether a reduced population size (30 animals) or the addition of a *myo-2p* fluorescent transgene
336 would impact the aggregation behaviors of each species. Aggregation in both *nigoni* and
337 *monodelphis* were significantly reduced compared to *elegans* (15 *myo-2p*+ and 15 *myo-2p-*
338)(**Figure 4C**) and significantly different from one another, which is consistent with the 50 animal
339 aggregation assay results (**Figure 2**). Furthermore, the inclusion of the *myo-2p* transgene did not
340 impact *elegans npr-1*(*ad609*) aggregation behavior. Next, we mixed 15 *myo-2p*+ *elegans npr-*
341 *1(ad609)* social feeding controls with 15 *myo-2p- elegans, monodelphis,* or *nigoni* on a seeded
342 NGM plate and allowed the mixed populations to acclimate for 45 minutes. After the acclimation
343 period, the plates were imaged and assayed for aggregation behavior. We aimed to understand if
344 the mixing of two distinct populations would alter the aggregate feeding dynamics of either or both
345 species. In the mixed species population assays, we noted that addition of *nigoni* to the *myo-2p*+
346 *elegans* population significantly reduced aggregation behavior of elegans, while with the addition
347 of *monodelphis* the reduction was not significant but trended strongly downward (**Figure 4D&E**).
348 Interestingly, we did not observe an impact of *elegans* on behavior of 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.



352 **Figure 4. Ecological drivers can modulate social feeding behaviors. (A)** Representative images
353 and **(B)** quantification of number of aggregating animals of *Caenorhabditis* species in mixed sex
354 populations. *tropicalis* and *monodelphis* both showed significant differences in aggregation when
355 males were added to the population. Bar graphs represent the mean values across biological
356 replicates (each replicate indicated by a dot). Error bars show stand error of mean. Standard T-test
357 was used for individual comparisons; p-values are indicated in standard format (p=0.05(*),
358 p=0.01(**), p=0.001(***), p= <0.001 (****)). Hermaphroditic species are labeled in red, while
359 gonochoristic species are labeled in black. **(C)** Quantification of 30 animal single-species controls.
360 **D)** Quantification of mixed species assays, where 15 *elegans myo-2p*+ animals were mixed with
361 15 *elegans myo-2p-, monodelphis,* or *nigoni*. Combining *elegans* and *nigoni* significantly lowers
362 aggregation of *myo-2* expressing *elegans* compared to their *elegans* alone control (left), while
363 addition of monodelphis caused an insignificant but trending reduction (left). *monodelphis* and
364 *nigoni* have significantly lower aggregation than their *elegans myo-2p*– control (right). **(E)**
365 Representative images of mixed species assays. *myo-2p* expressing *elegans* are indicated by arrows
366 (top left window).
367

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

369 Given the variation in the social feeding behaviors in the *Caenorhabditis* genus, we
370 wondered about mechanisms that might result in species differences. Morphological differences
371 likely drive some of the changes, however, there has been very little characterization in most of
372 the species we assayed. There are several papers examining the development of the vulva [33–35], tail
373 morphology[36–38] and body size[39], however there is still a large deficit in the literature. This lack of
374 species characterization is especially apparent when considering the nematode nervous systems,
375 with the only other *Caenorhabditis* species with any significant neuronal characterization being
376 *briggsae*[40,41]. To bypass the lack of circuit-based knowledge within the clade, we leveraged several
377 known genetic drivers of social feeding behavior in *elegans*, notably the neuropeptide receptor
378 NPR-1. To compare *npr-1* across species, we first queried both existing and draft genome
379 assemblies for orthologues using OrthoFinder[27]. Given the size and relative domain conservation
380 of the NPR family of genes in *elegans* we were somewhat surprised to identify a single orthologue
381 of NPR-1 in all species except for *nigoni*, which had multiple related paralogues and was thus
382 excluded from our analysis. To characterize sequence conservation among species we used
383 CLUSTAL V5.0 analysis to perform a multiple sequence alignment to produce an NPR-1
384 alignment (**Figure 5A**). The alignment showed there is a high level of conservation among the
385 species, even when considering the most evolutionary divergent species, like *monodelphis*. We
386 queried three residues in NPR-1 that when mutated have been shown to induce social behaviors[12]
387 and found them to be well conserved and wildtype. Interestingly, when compared to a consistent

protein length among species, *uteleia* also appears to have a large expansion of its *C-terminal* tail to include a novel Hox DNA-binding homeodomain. **(Figure 5B)**. However, given the draft nature of the *uteleia* genome assembly, this expansion could also be an artifact of an adjacent or overlapping gene feature.

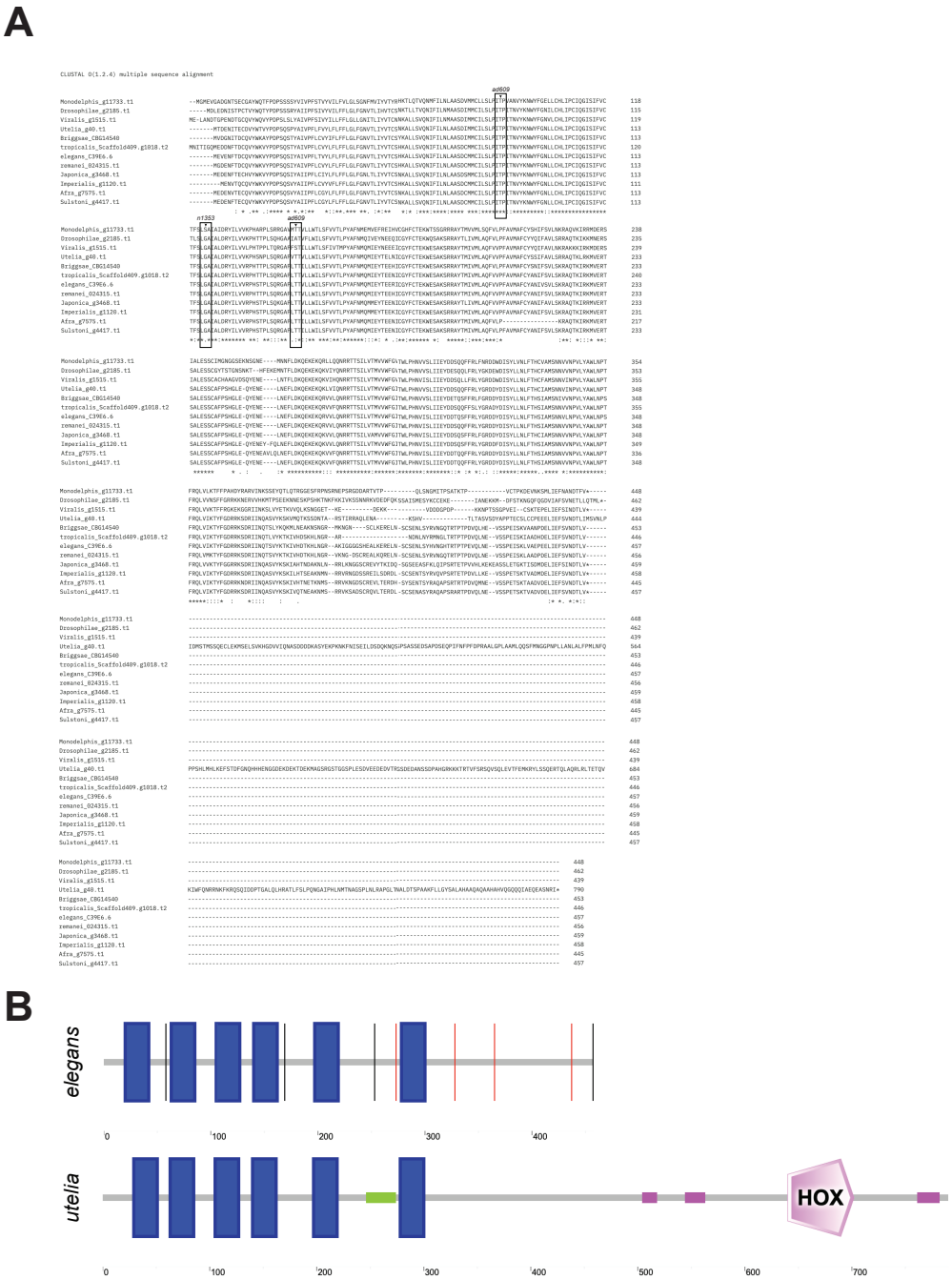


Figure 5. Protein alignment of NPR-1 orthologues in amongst *Caenorhabditis* nematode species. **(A)** NPR-1 orthologues were queried from existing *Caenorhabditis* genomes or assemblies based on sequence similarity. A single orthologue was identified in each species with the exception of *nigoni* which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

397 NPR-1 is relatively well conserved across the clade, with the exception of *uteleia* which showed a
398 large expansion of amino acid sequence to include a novel Hox homeodomain **(B)**.
399

400 **Discussion**

401 Here, we examined several social foraging behaviors of an evolutionarily diverse clade of
402 *Caenorhabditis* nematodes to better understand how such behaviors differ and may have evolved
403 between species. Across these divergent species of nematodes, we noted several surprising trends
404 within a set of social feeding behaviors that that underpin a shared general feeding strategy.
405 Notably, we confirmed of *elegans* (*npr-1*) is a relative extreme when compared to other species
406 but can be partially phenocopied by several wild isolates and other species. In contrast, most of
407 the species we assayed (including the wild *elegans* isolate CB4856 'Hawaiian' strain) showed
408 intermediate levels of aggregation, while basal taxon *monodelphis* showing little to no aggregation.
409 Furthermore, we uncovered a surprising trend when examining bordering behavior among species,
410 notably that the phenotype seems to generally decline the further away the species is in relatedness
411 to *elegans*. While this trend is not perfect, it does again highlight the idea that N2 'Bristol' *elegans*
412 may represent a behavioral outlier when compared with wild *elegans* isolates and most other
413 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 nuanced genetic heterogeneity present between individuals, maintained in part through
419 selection pressure. *elegans* strains have been artificially selected and lab-evolved (in part because
420 they 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

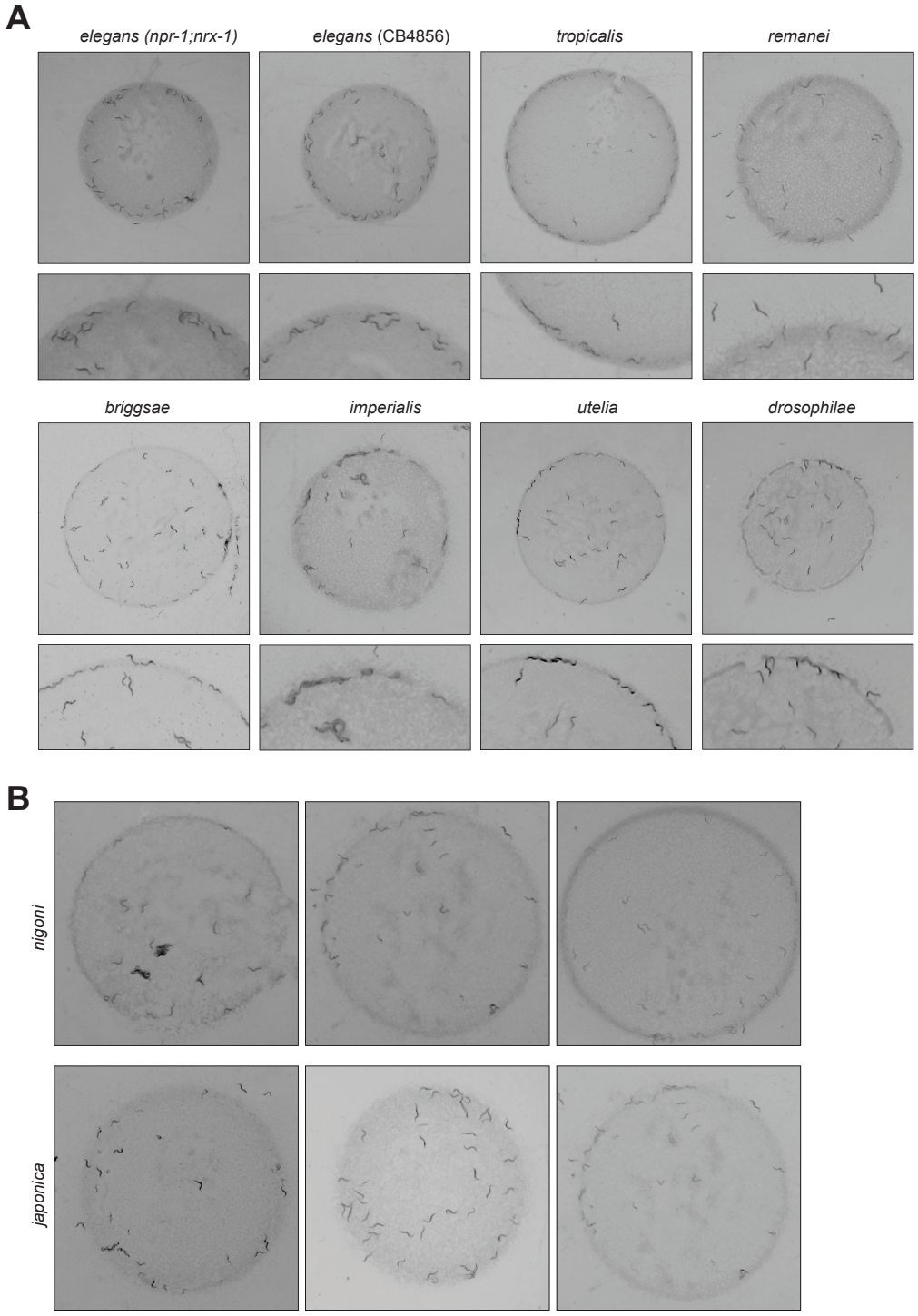
429 guanine substitution in NPR-1 is sufficient to produce a gain of function phenotype, eliminating
430 social behavior and inducing solitary feeding[12]. Lastly, it is not unreasonable to speculate that
431 SNPs present within individuals may have a modulatory effect within particular behavioral
432 circuits, especially if they are closely associated with critical neuronal genes or regulatory
433 elements. This concept is highlighted in recent work showing mutations in the *daf-19* transcription
434 factor are able to dramatically alter chemosensory and oxygen-sensing circuits leading to a
435 disruption social feeding behaviors in both *C. elegans* and *P. pacificius*[42].

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 alter if those conditions were to
445 more accurately reflect what occurs in the wild.

446 Furthermore, the substate 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 substates. 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 substates 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[43]. 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[43,44]. 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[45,46]. 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 if

460 strongly diminished[13], likely as part of a co-dependent circuit detecting both starvation cues and
461 increased oxygen[47].
462 The last major factor potentially influencing these behaviors is chemical signaling in the
463 form of environmental secretions and touch of other animals. Although the nature of these
464 chemical modulators is widely unknown, they represent an intriguing mechanism by which
465 individuals can modulate interactions with other animals and their environment. For example, it is
466 well known that several species of nematodes have complex and diverse secretomes, which they
467 use to modulate interactions with their environment. Several studies have shown that parasitic
468 nematodes secrete protein or chemical modulators to downregulate their host's immune response[48].
469 Similarly, the *elegans* genome encodes a large number of putative secreted proteins[49], some of
470 which were isolated via mass spectrometry, identifying a number of novel and nematode specific
471 protein factors of unknown function. In parallel, the chemical secretome of *elegans* revealed a
472 complex biochemical landscape, heavily enriched in putative pheromones, especially ascaosides[50].
473 Given the unknown function of many of these proteins and chemicals it is not unreasonable to
474 assume at least a subset may be influencing the behaviors of both individuals and populations of
475 individuals. Several ascaroside pheromones for example are well documented to alter population
476 behaviors, modulating hermaphrodite-male attractions and repulsion, mating, and dauer formation
477 in the absence of food[51]. Furthermore, pheromone secretion detected in the environment can also
478 induce the production of EV's in neuronal tissues of the affected animals, potentially altering
479 behavior[52]. Taken together we must consider that unknown signaling molecules are modulating
480 the interaction between individuals in our social feeding assays. Among the individuals of the same
481 species, these signals may provide a real-time readout of the local sensory environment helping
482 animals to respond and alter their behavior accordingly. Among individuals of different species,
483 the 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, the variation we observed among
488 species across several behavioral paradigms in not altogether surprising. 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 identify/density[13,43,44], and O_2/CO_2 balance[53].

491 Complicating matters is recent evidence in *P. pacificus* showing that even within an established
492 circuit single cues such as elevation (and corresponding depletion of O_2) can override the
493 established behavioral paradigm and introduce unanticipated variation[14]. While the idea of
494 context-dependent variation is likely to complicate dissection of the genetic and molecular
495 mechanisms of behavioral variation, it also presents and exciting opportunity to study real-time
496 plasticity within neuronal circuits.



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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