

1 **Transcriptional signatures of invasiveness in *Meloidogyne*** 2 ***incognita* populations from sub-Saharan Africa**

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10

11 **Abstract**

12 *Meloidogyne incognita* is an economically important plant parasitic nematode. Here we
13 demonstrate substantial variation in the invasiveness of four *M. incognita* populations relative to
14 tomato. Infective (J2) stage transcriptomes reveal significant variation in the expression of protein-
15 coding and non-coding RNAs between populations. We identify 33 gene expression markers (GEMs)
16 that correlate with invasiveness, and which map to genes with predicted roles in host-finding and
17 invasion, including neuropeptides, ion channels, GPCRs, cell wall-degrading enzymes and microRNAs.
18 These data demonstrate a surprising diversity in microRNA complements between populations, and
19 identify GEMs for invasiveness of *M. incognita* for the first time.

20

21 Key words: Root-knot nematode, behaviour, invasion, transcriptome, microRNAs, plant parasitic
22 nematode.

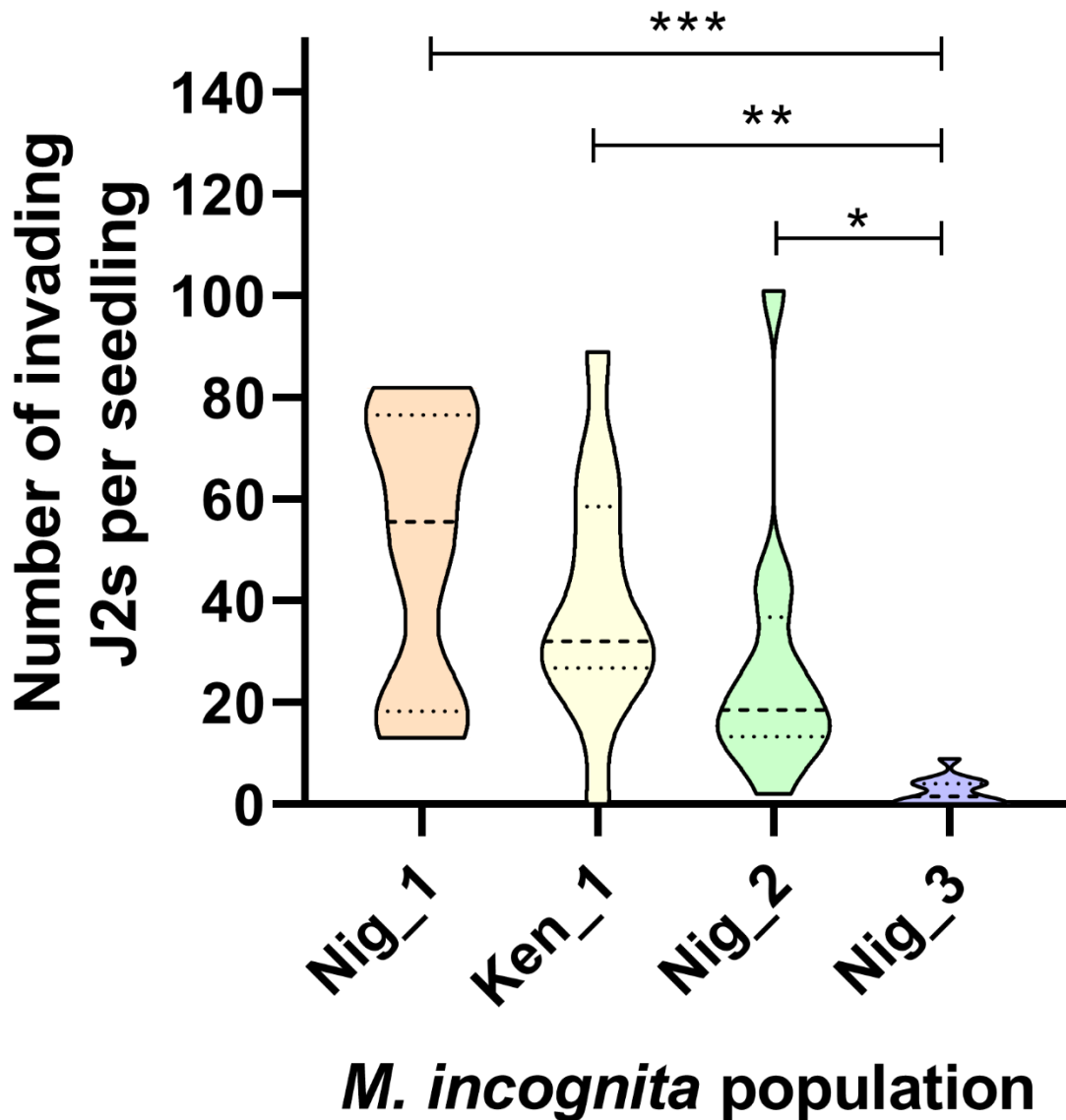
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24 *Meloidogyne incognita* is a globally distributed and highly polyphagous parasite of crop plants
25 (Coyne et al., 2018; Trudgill & Blok, 2001), demonstrating a surprisingly high level of adaptive
26 variability for an asexual organism (Szitenberg et al., 2017). This adaptability is thought to play a role
27 in the pests' ability to rapidly evade sources of crop resistance. Consequently, *M. incognita* is
28 becoming increasingly problematic, and current approaches to control are insufficiently robust or
29 durable to provide reliable protection in the field (Davies & Elling, 2015). The natural variation
30 between *M. incognita* populations is poorly understood (Bucki et al., 2017). This constitutes a
31 substantial gap in our knowledge, which could hinder our ability to develop sources of durable
32 resistance to field populations. The relatively high work burden of population maintenance in the
33 laboratory, and inevitable domestication of *M. incognita* populations makes the assessment of field-

34 relevant inter-species variation a significant and ongoing technical challenge. In addition, access to
35 populations that are native to Nagoya protocol (<https://www.cbd.int/abs/>) signatories can be
36 problematic. Whilst the Nagoya protocol aims to promote equitable commercial outcomes arising
37 from native genetic resources, opportunities for collaboration and extended sharing of resources are
38 limited. Collectively, these challenges promote an artificial over-reliance on highly domesticated
39 legacy strains, which are unlikely to reflect the genotypic or phenotypic spectra of field populations.

40 Although there are many potential approaches to developing crop parasite resistance, an
41 improved understanding of parasite host-finding and invasion may facilitate the development of
42 new strategies that prevent infection. This is preferable to sources of resistance that are active *in*
43 *planta*, as it limits the opportunity for secondary pathogen infection, and minimises the metabolic
44 burden of mounting a defence response to invading parasites. In this study, we assessed the host-
45 finding and invasion behaviour of *M. incognita* populations that had been recently collected from
46 field sites in Kenya and Nigeria, with the aim to relate observed behavioural variation to gene
47 expression signatures using transcriptomic correlation. These data would improve our
48 understanding of the link between genotype and phenotype, which may enable us to identify new
49 targets for nematicide development, or biotechnological intervention.

50 We considered three populations collected from Nigeria, named Nig_1, Nig_2, and Nig_3,
51 and one population from Kenya, named Ken_1. Our data demonstrate statistically significant
52 variation in the propensity of these *M. incognita* populations to invade tomato cv. Moneymaker
53 seedlings. Nig_1 is the most invasive, with a mean of 50.06 ± 7.9 J2s (from a total of 200 J2s) invading
54 within 24 h, followed by Ken_1 with a mean of 39.9 ± 6.9 , Nig_2 with a mean 27.3 ± 7.6 , and Nig_3
55 being the least invasive, with a mean of 2.5 ± 0.8 J2s invading within 24 h (Figure 1).



56

57 **Figure 1. *Meloidogyne incognita* invasion of tomato cv. Moneymaker seedlings is highly variable.**

58 Violin plot showing number of J2s invading tomato seedlings, 24 h post exposure. Dashed lines

59 indicate the median, dotted lines indicate the quartiles. Data assessed by ANOVA and Tukey's

60 multiple comparison test using Graphpad Prism 8; $P < 0.05^*$, $P < 0.01^{**}$, $< 0.001^{***}$. *M. incognita*

61 populations were collected from field sites in Kenya and Nigeria. They were cultured on tomato cv.

62 Moneymaker, in plant growth cabinets at 23°C, with a regular 16 h light, 8 h dark cycle for no more

63 than two generations following field collection. Tomato seedling infection assays were conducted as

64 in Warnock et al. (2016), using 200 J2s per seedling, inoculated into an agar slurry containing the

65 tomato seedling.

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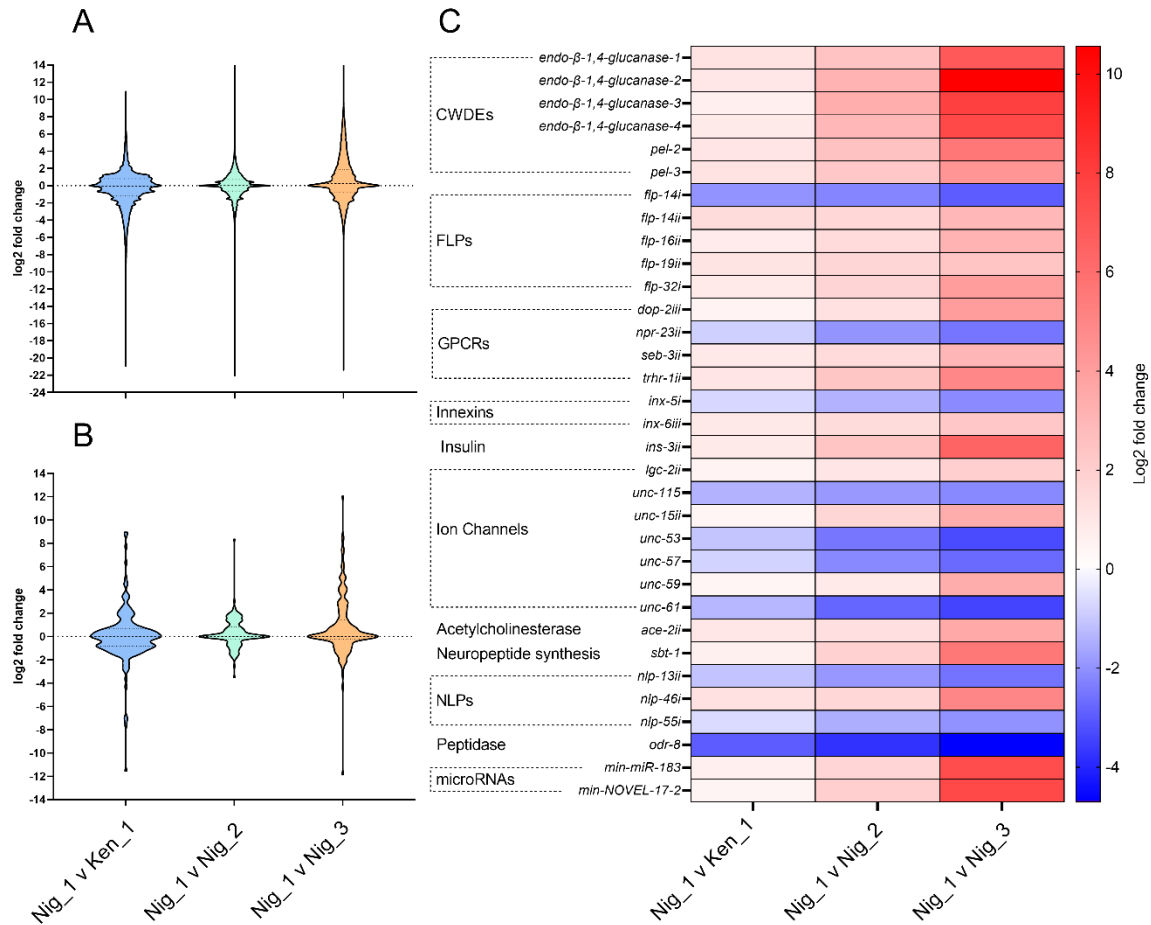
67 We conducted high-throughput sequencing of protein-coding and non-coding RNAs from the

68 infective J2 stage of each *M. incognita* population to understand the molecular basis of behavioural
69 variation. Our data revealed that up to 6,232 (13.7%) transcripts were significantly up-regulated
70 ($P < 0.0001$ ****) relative to the most invasive population, Nig_1, with up to 4,908 (10.8%) down-
71 regulated across pairwise comparisons (Figure 2A; supplemental file S1). Using mirDeep2, we
72 identified 192 precursor microRNA genes across the four *M. incognita* populations, relating to 144
73 predicted mature microRNAs in *M. incognita* Nig_1; 146 in Nig_2; 105 in Nig_3; and 176 in the Ken_1
74 population. This constitutes a surprising diversity in microRNA complement between populations of
75 the same species, with Ken_1 representing the major outlier, with 44 predicted mature microRNAs
76 unique to that population (supplemental file S2). By way of comparison, a similar analysis using the
77 entomopathogenic nematode *Steinernema carpocapsae* revealed variation from 269 to 273
78 predicted mature microRNAs across three populations (Warnock et al., 2018). Up to 52 (27%) of the
79 predicted and conserved *M. incognita* microRNA genes were significantly up-regulated
80 ($P < 0.0001$ ****) relative to the most invasive Nig_1 population, with up to 23 (12%) down-regulated
81 across pairwise comparisons (Figure 2B).

82 We populated a list of Gene Expression Markers (GEMs) that correlated, either positively or
83 negatively, with the observed invasion phenotypes. This was achieved by arranging the population
84 comparisons from most invasive to least invasive (Nig_1 vs Ken_1; Nig_1 vs Nig_2; Nig_1 vs Nig_3),
85 and constraining gene lists to those that followed expression patterns consistent with the
86 phenotypic trend. Correlating GEMs were identified when the log₂ fold change quotients between
87 adjacent comparisons were greater than one, with at least a $P < 0.05$ * difference between each
88 population, and at least $P < 0.0001$ *** between the most and least invasive populations. Using this
89 approach, we identified 485 GEMs that correlate with the observed invasion phenotype of *M.*
90 *incognita*, comprising 483 protein-coding genes, and two microRNA genes; 242 GEMs correlate
91 positively with the invasion phenotype, and 243 GEMs correlate negatively (Supplemental Files S1 &
92 S2). On inspection of the invasion GEM list, we identified a total of 33 genes with predicted roles in
93 the regulation of host-finding and invasion behaviour, including genes associated with the
94 neuropeptidergic system, neuronal signalling, cell wall-degrading enzymes, and the two microRNA
95 genes (Figure 2C). It is possible that other correlating genes play a functional role in the invasion
96 phenotype, however we deemed that these 33 genes were most likely to exert the largest influence,
97 based on known or predicted functionality.

98 Six neuropeptide genes correlated positively with *M. incognita* invasiveness, including
99 *FMRamide-like peptide 14ii (flp-14ii)*, *flp-16ii*, *flp-19ii*, *flp-32i INSulin-like protein 3ii (ins-3ii)*, and
100 *Neuropeptide-Like Protein 46i (nlp-46i)*. Three neuropeptide genes, *flp-14i*, *nlp-13i* and *nlp-55i* were
101 negatively correlated with the invasion phenotype (Figure 2C). Expression of a predicted

102 prohormone convertase chaperone, *sbt-1*, which is required for the biosynthesis of neuropeptides in
103 the free-living nematode *Caenorhabditis elegans* (Husson & Schoofs, 2007), also correlated positively
104 with invasion behaviour. These data implicate the neuropeptidergic system, and FLPs in particular, in
105 the modulation of *M. incognita* invasion behaviour. This corroborates previous observations of *flp*
106 gene enrichment within the infective juvenile stage of many parasitic nematode species, and a role
107 in the behavioural diversification of these stages (Lee et al., 2017). Indeed, our own work
108 demonstrates similar associations between neuropeptidergic genes and the host-finding behaviour
109 of *S. carpocapsae* (Warnock et al., 2018; Morris et al., 2017). Four putative neuropeptide G-Protein
110 Coupled Receptor (GPCR) genes were also found to associate with *M. incognita* invasiveness, along
111 with seven ion channel genes, two innexin genes, an acetylcholinesterase and a predicted *odr-8*
112 peptidase homologue (Figure 2C). Within the 485 correlating GEMs, we also identified 62 novel
113 genes, with no known function, or orthology to *C. elegans* genes (supplemental file S1). Six plant Cell
114 Wall-Degrading Enzyme (CWDE) genes were also associated positively with invasion phenotypes,
115 corresponding to four *endo- β -1,4-glucanase* genes, and two predicted pectate lyase (*pel*) genes
116 (Figure 2C). Each CWDE gene is most highly expressed in the most invasive Nig_1 population, and
117 display lowest expression in the least invasive population, consistent with a role in mediating the
118 enzymatic degradation of the plant cell wall. If it can be demonstrated that certain CWDEs confer a
119 specific advantage for the invasion of particular host species, it could point to new approaches to
120 resistance based on the modification of cell wall composition, potentially in conjunction with recent
121 developments in synthetic biology.



M. incognita population comparisons

122

123 **Figure 2. Transcriptomic signatures of *Meloidogyne incognita* invasiveness.** Violin plots of log₂ fold

124 changes across pairwise population comparisons for (A) protein-coding genes and (B) microRNAs. (C)

125 Summary heatmap of 33 selected GEMs, demonstrating the log₂ fold change between pairwise

126 comparisons, relative to the most invasive Nig_1 population. GEMs follow a gradient expression

127 pattern that positively or negatively correlates with the population invasion phenotype; CWDEs –

128 Cell Wall Degrading Enzymes. Figures were generated in Graphpad Prism 8. RNA extraction, library

129 preparation, sequencing, bioinformatics and statistical analyses were conducted as in Warnock et al.

130 (2018). Briefly, ~3000 J2s of each *M. incognita* population were used to extract total RNA, from

131 which coupled 150 bp paired-end, and 50 bp single end illumina HiSeq libraries were prepared for

132 each population, in triplicate. Libraries were sequenced on one illumina HiSeq 2500 lane. Following

133 quality control, reads were mapped to the most recent *M. incognita* genome assembly (PRJEB8714,

134 WBPS12, <https://parasite.wormbase.org>) using STAR and RSEM (Blanc-Mathieu et al., 2017; Howe et

135 al., 2015; Dobin et al., 2013; Li et al., 2011). MicroRNAs were identified and quantified using

136 MirDeep2 (Friedländer et al., 2012). Predicted microRNAs were named using a BLAST search against

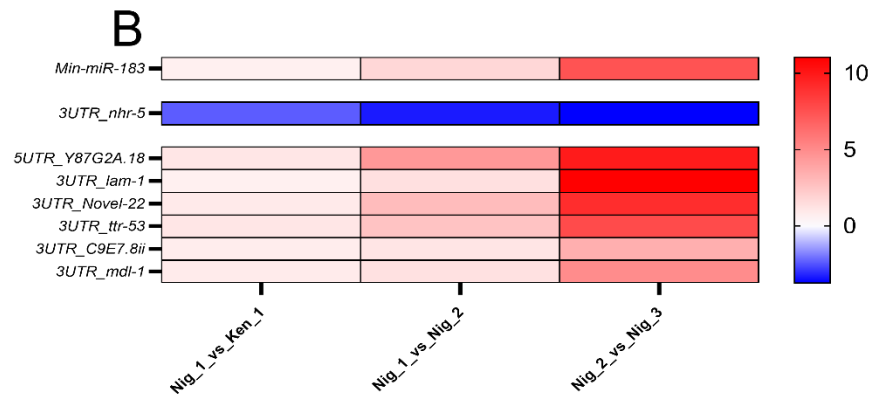
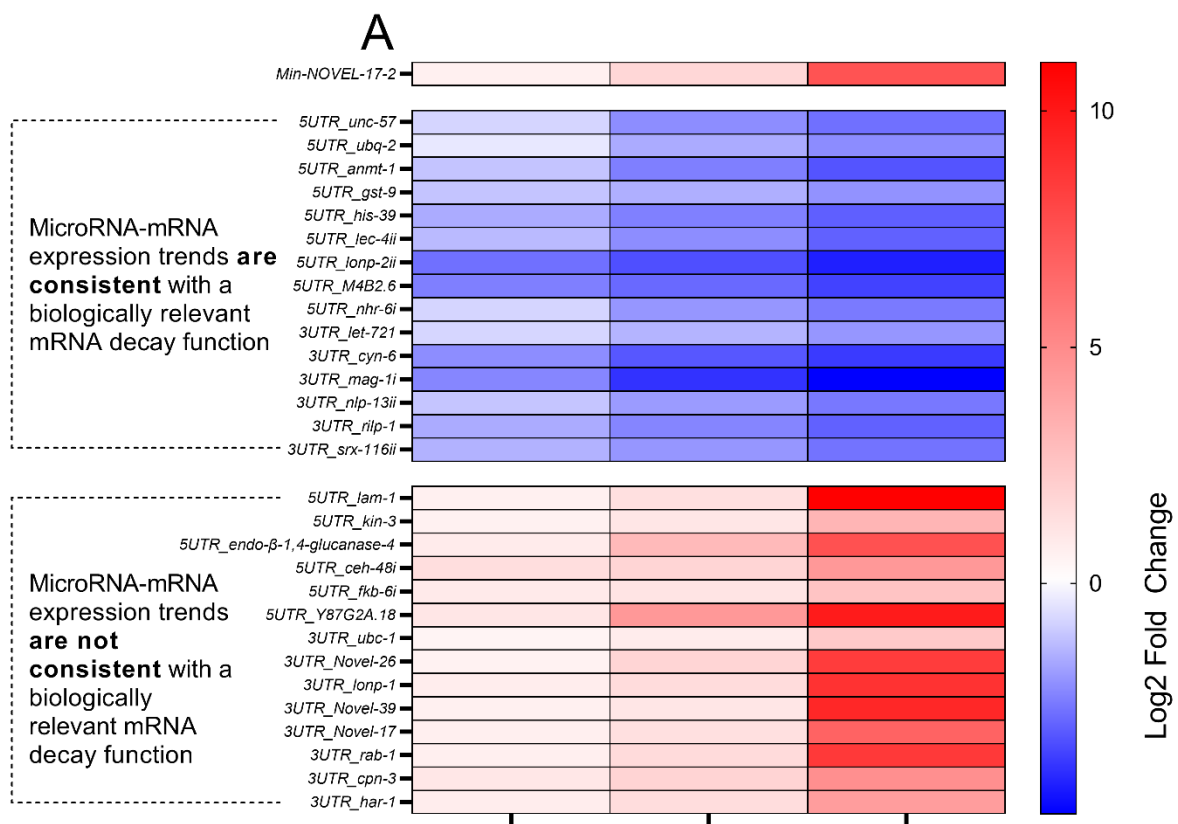
137 *C. elegans* microRNAs (www.miRBase.org), and previously identified *M. incognita* microRNAs (Zhang

138 et al., 2015). Predicted microRNAs were named in line with *C. elegans* or *M. incognita* microRNAs
139 represented as the top BLAST return, and if there was a sequence identity match greater than 80%.
140 All novel *M. incognita* microRNAs were named sequentially, ensuring no overlap with names
141 allocated to *C. elegans* or previously published *M. incognita* microRNAs. Predicted microRNA target
142 genes were identified with MiRanda (Enright et al., 2003), using strict and unrestricted discovery
143 modes, as in Warnock et al. (2018). Differentially expressed protein-coding and non-coding genes
144 were identified using DESeq2 (Love et al., 2014). All sequencing datasets are available from the SRA
145 database (Bioproject: PRJNA525879).

146

147 Non-coding microRNAs have been implicated in nematode behavioural variation (Warnock et al.,
148 2018; Rauthan et al., 2017; reviewed in Ambros & Ruvkun, 2018), through the regulation of target
149 gene expression. Our data reveal that the expression of two mature microRNAs correlates with the
150 invasion phenotype of *M. incognita* populations (Figure 2C). Using miRanda to identify predicted
151 gene targets, in both strict and unrestricted settings, reveals a surprising abundance, and inter-
152 connection between these microRNAs and neuropeptide genes, spanning the *flp*, *nlp* and *ins*
153 families, in addition to GPCR and ion channel genes (supplemental file S3, S4). It has been suggested
154 that microRNAs regulate developmental programmes through the coordinated and cooperative
155 targeting of genes involved in specific biological functions (Zhang et al., 2009). Our data indicate that
156 this may also be the case for behavioural regulation. For example, *Min-NOVEL-17-2* is predicted to
157 simultaneously target: *flp-1i*, *flp-1ii*, *flp-11i*, *flp-11ii*, *flp-11iii*, *flp-33i*, *flp-33ii*, *flp-34i*, *ins-1i*, *ins-1ii*, *ins-*
158 *1iv*, *ins-1v*, *ins-18i*, *nlp-8ii*, *nlp-12*, *nlp-13i*, *nlp-13ii*, *nlp-81iii*, in addition to a variety of other ion
159 channel, GPCR and innexin genes (supplemental file S5). To further investigate the potential
160 relationship between microRNAs and behavioural regulation, our analysis sought to identify
161 predicted interactions that followed the expected trend for biologically interacting microRNA-
162 mRNAs, at the level of mRNA abundance. A substantial literature has developed around microRNA
163 induced mRNA decay in animals (reviewed in Iwakawa and Tomari, 2015), and our data demonstrate
164 a correlation between numerous predicted microRNA targets, and expression patterns between
165 populations, indicating that these microRNA-mRNA interactions may be biologically relevant. For
166 example, *nlp-13ii* is identified as both an *in silico* predicted target of the novel microRNA *Min-*
167 *NOVEL-17-2* and is demonstrated to follow an expression pattern consistent with microRNA-
168 mediated mRNA decay across populations (Figure 3A). However, whilst *nlp-13i* is also a predicted
169 target of *Min-Novel-17-2*, it does not follow an expression trend that is consistent with microRNA-
170 mediated decay. This indicates either that the *nlp-13i* and *nlp-13ii* transcripts are expressed in
171 different cells / tissues that only partially overlap with expression of *Min-NOVEL-17-2*, or that there

172 are other transcript-specific features, which influence the tendency towards microRNA-mediated
 173 mRNA decay or translational inhibition. One possible explanation relates to altered secondary
 174 structure of UTR sequences, which may underpin differences in the bioavailability, or function of
 175 microRNA target sites. Based on *in silico* structural predictions using the Vienna RNAfold server
 176 (<http://rna.tbi.univie.ac.at/>), this does not appear to be a factor for the UTRs of *nlp-13* gene copies
 177 at least. Our analysis in Figure 3 focuses solely on predicted microRNA interactions with the 483
 178 protein-coding genes identified as invasion GEMs, and on that basis makes no judgement on the
 179 likelihood of interactions with the many other predicted target genes listed above, which do not
 180 follow the stringency criteria used to populate the list of GEMs.



M. incognita population comparisons

182 **Figure 3. Differential expression of predicted microRNA targets suggests biologically relevant**
183 **interactions across *Meloidogyne incognita* populations.** (A) Heatmap demonstrating differential
184 expression trends for the microRNA, *Min-NOVEL-17-2*, and predicted mRNA targets identified within
185 the list of 483 protein-coding GEMs. The location of the predicted microRNA interaction point is
186 indicated as a 5'UTR or 3'UTR suffix. 15 genes negatively correlate with *Min-NOVEL-17-2* changes
187 across pairwise population comparisons, which suggests biologically relevant interactions, mediated
188 through mRNA decay. 14 of the predicted targets, which follow the phenotype trend, do not
189 correlate with *Min-NOVEL-17-2* in a way that suggests a biologically relevant interaction. (B) Heatmap
190 demonstrating differential expression trends for the microRNA, *Min-miR-183* and predicted mRNA
191 targets identified within the list of 483 protein-coding GEMs. Only one of the predicted target genes
192 negatively correlates with microRNA differential expression trends, suggesting that the putative
193 nuclear hormone receptor gene, *nhr-5*, is the only biologically relevant target within this set of
194 invasion GEMs. The microRNA target analysis presented here is not intended to be an exhaustive
195 treatment of all predicted mRNA interactions, focusing instead on the GEMs identified through
196 transcriptomic and phenotypic correlation.
197
198 Apomictic *Meloidogyne* spp. are known to possess highly divergent hypotriploid genomes, with
199 multiple variant gene copies (Szitenberg et al., 2017). The sequence variation of gene copies may
200 reflect the functional diversification of common genetic elements for adaptive purposes, through
201 hybridisation and selection. For example, a significant number of putative neuropeptide gene copies
202 are found to encode the same predicted mature neuropeptide(s) within a variant mRNA sequence;
203 such genes are identified within this manuscript by virtue of a Roman numeral suffix, assigned
204 according to the order of discovery. Expression of *flp-14ii* correlates positively with increased
205 invasiveness, whereas *flp-14i* correlates negatively with increased invasion behaviour (Figure 3, and
206 Supplemental File S1). Whilst we have no insight to the relative role or function of gene copies, or if
207 these copies co-localise, we have established that they can be differentially expressed, within and
208 between populations of the same species. Our analysis of predicted microRNA interactions reveals a
209 considerable amount of variation in the predicted 5' and 3' UnTranslated Regions (UTRs) of
210 predicted neuropeptide gene copies, which underpins qualitative and quantitative variation in
211 predicted microRNA targeting (supplemental files S3, S4 and S5). UTR sequence variation has
212 received little attention in the literature for parasitic nematode species, however UTR sequences are
213 known to be highly variable across developmental stages, and tissues of the model *C. elegans*, which
214 drives the genic regulation of microRNA interactions (Blazie et al., 2010; Mangone et al., 2010). It is
215 possible that the hypervariation of gene copy UTRs between *M. incognita* populations could be

216 adaptive, driving functional divergence as a factor of differential microRNA targeting. Data support a
217 similar hypothesis for UTR isoform variation and behavioural diversification of *S. carpocapsae* strains
218 (Warnock et al., 2018). This could provide a functional explanation for the extraordinary variation
219 and adaptiveness of apomictic *Meloidogyne* spp.

220 This study demonstrates a surprising behavioural variation amongst *M. incognita*
221 populations that are native to Kenya and Nigeria and provides the first evidence of GEMs that
222 correlate with the invasion phenotype. Furthermore, we observe substantial variation in the
223 complement of microRNA genes between populations, and variation in gene UTR targets between
224 variant gene copies, which could underpin behavioural adaptation to host and environment. These
225 observations require detailed functional studies to ascertain the specific influence of implicated
226 genes and microRNAs. Whilst the inevitable domestication of *M. incognita* populations under
227 laboratory and greenhouse conditions constitutes a technical challenge for the study of field-
228 relevant diversity and phenotype, we expect these populations to become better adapted to the
229 experimental host. This could provide opportunity to track signatures of molecular adaptation over
230 time, within an experimental evolutionary approach (reviewed by Kawecki et al., 2012).

231

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234 and a GCRF pilot grant from the Department for the Economy, Northern Ireland. We would like to
235 thank Bastian Fromm (Stockholm University) for discussions around UTR secondary structure as a
236 potential driver of microRNA interactions.

237

238 **Supporting information captions**

239

240 Supplemental file S1. Global DESeq2 output across all pairwise population comparisons, and
241 complete list of invasion GEMs.

242 Supplemental file S2. List of predicted microRNAs and global DESeq2 output across all pairwise
243 population comparisons.

244 Supplemental file S3. MicroRNA target prediction analysis for global 5'UTRs.

245 Supplemental file S4. MicroRNA target prediction analysis for global 3'UTRs.

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