1 **TITLE**

2 Cytoplasmic-nuclear incompatibility between wild-isolates of *Caenorhabditis nouraguensis*

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ABSTRACT

How species arise is a fundamental question in biology. Species can be defined as populations of interbreeding individuals that are reproductively isolated from other such populations. Therefore, understanding how reproductive barriers evolve between populations is essential for understanding the process of speciation. Hybrid incompatibility (e.g. hybrid sterility and lethality) is a common and strong reproductive barrier in nature, but few studies have molecularly identified its genetic basis. Here we report a lethal incompatibility between two wild-isolates of the nematode *Caenorhabditis nouraguensis*. Hybrid inviability results from the incompatibility between a maternally inherited cytoplasmic factor from each strain and a recessive nuclear locus from the other. We have excluded the possibility that maternally inherited endosymbiotic bacteria cause the incompatibility by treating both strains with tetracycline and show that hybrid death is unaffected. Furthermore, cytoplasmic-nuclear incompatibility commonly occurs between other wild-isolates, indicating that this is a significant reproductive barrier within *C. nouraguensis*. We hypothesize that the maternally inherited cytoplasmic factor is the mitochondrial genome and that mitochondrial dysfunction underlies hybrid death. This system has the potential to shed light on the dynamics of divergent mitochondrial-nuclear coevolution and its role in promoting speciation.

INTRODUCTION

How species arise is a fundamental and still unanswered question in biology. Under the biological species concept, species consist of populations of interbreeding individuals that are reproductively isolated from other such populations (Mayr 1942). Thus, to understand speciation, we must learn how reproductive barriers evolve between populations. Post-zygotic reproductive barriers are commonly found in nature, and occur when hybrid progeny are relatively unfit in comparison to their parents and serve as inefficient bridges for gene flow between populations. Hybrids can be extrinsically unfit, in that they are maladapted to their environment (e.g. hybrids exhibit an intermediate phenotype which is unfit in parental environments) or intrinsically unfit, in that they are developmentally abnormal (e.g. hybrids are sterile or inviable) (Coyne and Orr 2004).

The Bateson-Dobzhansky-Muller (BDM) model hypothesizes that hybrids are intrinsically unfit due to incompatible gene combinations. In its simplest form, the model predicts that at least two genetic loci, each having evolved independently in one of two divergent lineages, have deleterious epistatic interactions in hybrids. This model has gained support by the molecular identification of genes required for hybrid dysfunction in several genera (Presgraves 2010). Identifying these genes and the natural forces that drive their evolution is one of the major objectives of speciation genetics. Darwin suggested that differential ecological adaptation by natural selection was the major driving force for speciation. Some of the molecularly identified "incompatibility genes" do indeed show signs of selection (Ting 1998; Presgraves *et al.* 2003; Barbash *et al.* 2004; Brideau *et al.* 2006; Oliver *et al.* 2009; Chae *et al.* 2014; Phadnis *et al.* 2015), but do not always have a clear role in promoting ecological adaptation (Tao *et al.* 2001; Ferree and Barbash 2009; Phadnis and Orr 2009; Seidel *et al.* 2011). However, there are currently only a handful of known incompatibility genes from a limited number of genera. Additional studies from a wider range of taxa are needed to gain a better understanding of the evolutionary forces that drive speciation.

65 Some studies on the genetic basis of hybrid incompatibility have focused on strong post-

66 zygotic reproductive barriers between well-defined species. These studies reveal that many

67 genetic variants contribute to dysfunction of hybrids (Coyne and Orr 1998), but provide little insight

68 into the dynamics of the accumulation of such variants or their relative roles in initiating speciation.

69 For example, theoretical work indicates that the number of genetic incompatibilities increases

70 greater than linearly with the number of genetic differences between two lineages (Orr 1995).

71 Therefore, a small number of genetic incompatibilities may initially reduce gene flow and promote

72 genetic divergence between populations, while others evolve after strong reproductive barriers

73 have already been established. Given this, studies of incomplete post-zygotic barriers between

74 young species or divergent populations within species are essential to understand the

75 evolutionary forces that may initiate speciation.

76 Despite the paucity of molecularly identified incompatibility genes, the segregation of

77 deleterious phenotypes in a number of interspecific hybridizations indicates that incompatibilities

78 between cytoplasmic and nuclear genomes occur frequently (Ellison and Burton 2008; Ellison *et*

79 *al.* 2008; Sambatti *et al.* 2008; Arnqvist *et al.* 2010; Ross *et al.* 2011; Aalto *et al.* 2013).

80 Furthermore, several studies have definitively mapped these incompatibility loci to the

81 mitochondrial genome and nuclear genes with mitochondrial functions (Lee *et al.* 2008; Chou *et*

82 *al.* 2010; Luo *et al.* 2013; Meiklejohn *et al.* 2013; Huang *et al.* 2015). Aerobic eukaryotic organisms

83 rely on mitochondria to generate energy required for diverse biological processes. The

84 mitochondrial genome encodes a small fraction of mitochondrial proteins, but nuclear genes are

85 required for proper replication, transcription, and translation of mtDNA as well as other

86 mitochondrial proteins (Gustafsson *et al.* 2016). Given the interdependence of the nuclear and

87 mitochondrial genomes, they are expected to coevolve by the accumulation of compatible

88 mutations that maintain mitochondrial function. By extension, distinct lineages that undergo unique

89 mitochondrial-nuclear coevolution may be incompatible and result in mitochondrial dysfunction.

90 Several theories have been proposed to explain what drives the rapid coevolution of these two

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91   genomes, including adaptation to different carbon sources (Lee *et al.* 2008), arms races between

92   the genomes caused by genetic conflict over the relative fitness of males and females (Fujii *et al.*

93   2011), and the accumulation of deleterious mtDNA mutations and the evolution of compensatory

94   nuclear variants that rescue mitochondrial function (Rand *et al.* 2004; Oliveira *et al.* 2008; Osada

95   and Akashi 2012). However, given the scarcity of molecularly identified cases of mitochondrial-

96   nuclear incompatibilities, additional studies are required to form more complete theories regarding

97   the forces that drive their evolution.

98   Here we report incompatibility between the cytoplasmic and nuclear genomes of two

99   distinct wild-isolates of the male-female nematode *Caenorhabditis nouraguensis*. Cytoplasmic-

100   nuclear incompatibility is not specific to these two strains, but is also observed upon hybridization

101   of other distinct wild-isolates of *C. nouraguensis*, indicating that this is a naturally widespread

102   reproductive barrier within the species. The cytoplasmic-nuclear incompatibility we identify

103   between strains of *C. nouraguensis* may provide an excellent opportunity for a detailed study of

104   mitochondrial-nuclear incompatibility, the forces that drive the coevolution of these genomes, and

105   their possible role in speciation.

106

MATERIALS AND METHODS

Strain isolation and maintenance

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109   All strains of *C. nouraguensis* used in this study were derived from single gravid females

110   isolated in 2009 or 2011 from rotten fruit or flowers found in French Guiana (Kiontke *et al.* 2011),

111   and have not been subjected to further inbreeding. Strains were kindly provided by Marie-Anne

112   Felix ("JU" prefix) and Christian Braendle ("NIC" prefix). Strain stocks were stored at -80°C.

113   Thawed strains were maintained at 25°C on standard NGM plates spread with a thin lawn of OP50

114   bacteria (Brenner 1974).

115

Hybridizing JU1825 and NIC59

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117   To quantify inviability, we crossed one virgin L4 female and male, with 10-15 replicates for

118   each cross. The edge of each plate was coated with a palmitic acid solution (10 mg/mL in 95%

119   ethanol) and allowed to air dry, resulting in a physical barrier that helps prevent worms from

120   leaving the plate's surface. The plates were placed at 25°C overnight, during which the worms

121   matured to adulthood and began mating. The next day, each female-male couple was placed onto

122   a new plate streaked with OP50 and rimmed with palmitic acid. Each couple was then allowed to

123   mate and lay eggs for 5 hours at 25°C, and then were permanently removed. The embryos laid

124   within those 5 hours were counted immediately. Approximately 17 hours later, we counted the

125   number of embryos that failed to hatch per plate. These unhatched embryos were scored as dead

126   since *C. nouraguensis* embryogenesis is normally completed within 13 hours at 25°C (data not

127   shown). We defined the percentage of embryonic lethality as the number of unhatched embryos

128   divided by the total number of embryos laid. Approximately 20 hours later, we placed the plates at

129   4°C for an hour and then counted the number of healthy L4 larvae and young adults per plate. We

130   defined the percentage of viable progeny as the total number of L4 larvae and young adults

131   divided by the total number of embryos laid.

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Determining cytoplasmic-nuclear compatibility between various strains of C. nouraguensis

To test for an incompatibility between one strain's cytoplasm and another strain's nuclear genome, we compared the viabilities of backcrosses that differ only in the F1 hybrid female's cytoplasmic genotype (e.g. (NIC59); NIC59/JU1837 F1 female x JU1837 male vs (JU1837); NIC59/JU1837 F1 female x JU1837 male, Figure 3A). The genotype is designated by the following nomenclature: (cytoplasmic genotype); nuclear genotype. The cytoplasmic genotype indicates genetic elements that are only maternally inherited, such as the mitochondrial genome. We performed a Fisher's exact test to determine whether there were significant differences in the proportions of viable and inviable F2 progeny between the two types of crosses. We also calculated the relative viability of the two crosses (e.g. the percent viability of the (NIC59); NIC59/JU1837 F1 female x JU1837 male cross divided by the percent viability of (JU1837); NIC59/JU1837 F1 female x JU1837 male cross). Cytoplasmic-nuclear combinations that show a statistically significant difference in viabilities between the two types of crosses and a relative viability <1 were considered to be cytoplasmic-nuclear incompatibilities. Three biological replicates were performed for each cytoplasmic-nuclear combination except for JU1825 cytoplasmic - NIC24 nuclear and JU1825 cytoplasmic - NIC54 nuclear, which have four replicates each. For each biological replicate, 10 F1 hybrid L4 females were crossed to 10 L4 males on the same plate overnight at 25°C. The next day, they were moved to a new plate and allowed to lay embryos at 25°C for 1 hour. The parents were then removed and the percent viable progeny and embryonic lethality were calculated as described in the previous section of the Materials and Methods. The heat map used to visualize the median relative viability for each cytoplasmic nuclear combination was made using the heatmap.2 function from the gplot package in R.

Molecular Methods

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157   To determine if either JU1825 or NIC59 are infected with *Wolbachia*, we performed PCR on

158   crude lysates of both strains using degenerate primers targeted against two genes that are

159   conserved in *Wolbachia* (Baldo *et al.* 2006). Specifically, we attempted to detect *gatB* (gatB_F1

160   with M13 adapter, TGTAAAACGACGGCCAGTGAKTTAAAYCGYGCAGGBGTT, and gatB_R1

161   with M13 adapter, CAGGAAACAGCTATGACCTGGYAAYTCRGGYAAAGATGA) and *fbpA*

162   (fbpA_F3, GTTAACCCTGATGCYYAYGAYCC, and fbpA_R3,

163   TCTACTTCCTTYGAYTCDCCRCC). As controls, we performed PCR on squash preps of

164   *Drosophila melanogaster* w[1118] mutant strains (Bloomington stock number 3605) that were

165   infected or not infected with *Wolbachia*. *Drosophila melanogaster* strains were kindly provided by

166   the laboratories of Harmit Malik and Leo Pallanck.

167

168   ***Tetracycline treatment of JU1825 and NIC59***

169   Both JU1825 and NIC59 were passaged on 50 ug/mL tetracycline NGM plates streaked

170   with OP50 for nine generations. Both strains were treated by placing 10 L4 females and 10 L4

171   males on a fresh tetracycline plate and allowing them to mate and produce the next generation of

172   L4 progeny, which were then moved to a fresh tetracycline plate. Tetracycline plates were made

173   by allowing NGM plates with OP50 lawns to soak up a mixture of tetracycline and 1x M9. The

174   plates were left uncovered at room temperature until dry, and then used the following day.

175

176   ***Statistics***

177   P values were determined using R (v 3.2.5). Several statistical tests were used (Kruskal-Wallis

178   test followed by Dunn's test, and Fisher's exact test). When we performed several comparisons on

179   the same dataset, we used the Bonferroni method to correct p-values for multiple testing. Most

180   plots were made using the ggplot2 package in R.

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182   ***Data Availability***

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183   The authors state that all data necessary for confirming the conclusions presented in the article

184   are represented fully within the article and Supplemental Material.

185

RESULTS

Two strains of *C. nouraguensis* exhibit F2 hybrid breakdown

Two strains of *C. nouraguensis*, JU1825 and NIC59, were derived from single gravid

females that were isolated approximately 112 kilometers apart in French Guiana (Kiontke *et al.*

2011). Both of these strains were designated as belonging to *C. nouraguensis* based on having

highly similar ITS2 rDNA sequences, which serves as a good species barcode within the

Caenorhabditis genus, and because they produced many viable F1 offspring when crossed

(Kiontke *et al.* 2011; Félix *et al.* 2014). We found that both strains produce high numbers of viable

progeny in intra-strain crosses. We have also confirmed the previous finding of F1 hybrid viability

by crossing NIC59 females to JU1825 males, and vice versa, showing that the F1 hybrids

resulting from these inter-strain crosses exhibit levels of viability comparable to those seen in

intra-strain crosses (Figure 1).

However, not all reproductive barriers act in the F1 generation. There are many cases of F2

hybrid breakdown, in which reduction of hybrid fitness begins to manifest itself in the F2

generation due to recessive incompatibility loci (Masly *et al.* 2006; Bikard *et al.* 2009; Stelkens *et

al.* 2015). To test for F2 hybrid inviability, we mated hybrid F1 siblings derived from either JU1825

female x NIC59 male crosses, or from NIC59 female x JU1825 male crosses, and assayed the F2

generation for reductions in fitness. These F1 hybrids are referred to as "(J); N/J" and "(N); N/J"

respectively, where the genotype is designated by the following nomenclature: (cytoplasmic

genotype); nuclear genotype. The cytoplasmic genotype indicates genetic elements that are only

maternally inherited, such as the mitochondrial genome. We found that both types of F1 sibling

crosses resulted in a significant decrease in the percentage of viable progeny, with on average

only 71% and 63% of F2 embryos maturing to the L4 or young adult stage (Figure 1). These

results indicate that there are divergent genomic loci between NIC59 and JU1825 that cause

inviability only when they become homozygous in F2 hybrids. Additionally, there is no difference in

211 sex-specific mortality in hybrids in comparison to intra-strain crosses which implies that these loci

212 are autosomally linked, as we show later (Figure 1).

213

Incompatibilities between cytoplasmic and nuclear genomes cause F2 inviability

215 To further understand the genetic architecture of hybrid breakdown between JU1825 and

216 NIC59, we tested whether maternally or paternally inherited factors are required for F2 inviability.

217 We reasoned that backcrossing F1 females to parental males would test whether maternal factors

218 are required for reduced hybrid fitness, while backcrossing F1 males to parental females would

219 test whether paternal factors are required. For example, backcrossing F1 hybrid females to

220 JU1825 males will result in an F2 population with a 50% chance of being heterozygous

221 (NIC59/JU1825) and a 50% chance of being homozygous (JU1825/JU1825) for any given

222 autosomal locus. Therefore, this cross will test for maternally deposited NIC59 factors that are

223 incompatible with homozygous JU1825 autosomal loci. The same logic can be applied to crosses

224 of F1 hybrid males to parental strain females.

225 All instances of backcrossing F1 hybrid males to parental strain females resulted in levels

226 of F2 viability similar to those observed in parental strains. Therefore, paternal factors do not have

227 a major effect on F2 inviability (Figure 2A). Only two crosses consistently resulted in significantly

228 reduced viability. The first is when (N); N/J F1 females were crossed to JU1825 males, with on

229 average only 36% of F2 hybrids maturing to the L4 or young adult stage. This cross implies that

230 there are maternally derived NIC59 factors distributed to F2 embryos, and these factors are

231 incompatible with recessive JU1825 nuclear loci. The second is when (J); N/J F1 females are

232 crossed to NIC59 males, with on average only 52% of the F2 hybrids maturing to the L4 or young

233 adult stage (Figure 2B). This cross implies that there are also maternally derived JU1825 factors

234 distributed to F2 embryos, and these factors are incompatible with recessive NIC59 nuclear loci.

235 The viability of (J); N/J F1 female x JU1825 male crosses can also be significantly reduced in

236 comparison to intra-strain crosses, but varies within and between experiments (Figure S1).

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237   Interestingly, the F1 female backcross experiments show that almost identical crosses,

238   which differ only in the cytoplasmic genotype of the F1 female, have significantly different rates of

239   F2 viability. For instance, (N); N/J F1 female x JU1825 male crosses consistently have

240   significantly lower F2 viability than (J); N/J F1 female x JU1825 male crosses (Figure 2, Figure

241   S1). Similarly, (J); N/J F1 female x NIC59 male crosses consistently have significantly lower F2

242   viability than (N); N/J F1 female x NIC59 male crosses (Figure 2). These F1 hybrid females are

243   expected to be genotypically identical at all nuclear loci, suggesting that something other than the

244   F1 nuclear genome encodes maternal factors that lead to F2 inviability. One possible model to

245   explain the differences in these backcrosses is that the mitochondrial genome is the maternally

246   inherited factor that is incompatible with recessive nuclear loci in the F2 generation. For example,

247   all F2 progeny from (N); N/J F1 female x JU1825 male crosses will inherit only NIC59 mtDNA,

248   which may be incompatible with nuclear loci homozygous (or hemizygous) for JU1825 alleles

249   resulting in inviability (Figure 6A). In comparison, all F2 progeny from (J); N/J F1 female x JU1825

250   male crosses will inherit only JU1825 mtDNA, which should be compatible with the JU1825

251   nuclear genome and therefore not result in the same inviability. The same logic can be applied to

252   the (J); N/J F1 female x NIC59 male and (N); N/J F1 female x NIC59 male crosses. Therefore, we

253   hypothesize that F2 inviability is the result of two distinct cytoplasmic-nuclear incompatibilities, one

254   between the NIC59 mitochondrial genome and recessive JU1825 nuclear loci, and another

255   between the JU1825 mitochondrial genome and recessive NIC59 nuclear loci.

256

257   **The nuclear incompatibility loci are linked to autosomes**

258   Nematodes commonly have an XX (female) and XO (male) sex determining mechanism

259   (Pires-daSilva 2007). The F1 hybrid female backcross experiments reveal that there is no

260   difference in sex-specific mortality in hybrids in comparison to intra-strain crosses (Figure 2B).

261   However, given the expected genotypes of their F2 populations, these backcrosses on their own

262   do not allow us to determine whether the nuclear incompatibility loci are autosomally or X-linked.

13

263 In the previous section, we concluded that the inviability of the F2 progeny derived from (N); N/J

264 F1 female x JU1825 male crosses is the result of a genetic incompatibility between the NIC59

265 mitochondrial genome and nuclear loci homozygous (or hemizygous) for JU1825 alleles. If this is

266 true, it is reasonable to assume that the same genetic incompatibility occurs in (N); N/J F1 female

267 x (N); N/J F1 male crosses (Figure 1). In this F1 sibling cross, if the JU1825 nuclear incompatibility

268 locus were autosomally linked, both sexes would suffer equal rates of inviability. However, if the

269 nuclear incompatibility locus were linked to the X-chromosome, then we would expect a significant

270 decrease in the proportion of viable males in comparison to intra-strain crosses (Figure S2).

271 However, we observe no significant difference in the proportion of viable males for the (N); N/J F1

272 female x (N); N/J F1 male cross (Figure 1). Therefore, given the data from the F1 female

273 backcrosses and the F1 sibling crosses, we conclude that the JU1825 nuclear incompatibility

274 locus is autosomally linked. A similar line of reasoning indicates that the NIC59 nuclear

275 incompatibility locus is also autosomally linked.

276

277 **Endosymbiotic bacteria do not cause hybrid inviability**

278 We hypothesize that mitochondrial genomes are responsible for the cytoplasmic

279 component of the hybrid incompatibility between NIC59 and JU1825. However, we also

280 considered whether endosymbiotic bacteria of the *Rickettsiales* order could be involved. Within

281 this order, bacteria of the *Wolbachia* genus are known to infect certain species of nematodes, and

282 are transmitted to host progeny through female gametes (Werren *et al.* 2008). Furthermore, hybrid

283 lethality in inter-strain and interspecies crosses is sometimes caused by infection with divergent

284 *Wolbachia* strains (Bourtzis *et al.* 1996; Bordenstein *et al.* 2001). However, we failed to detect

285 conserved genes typically used to genotype diverse strains of *Wolbachia* in either JU1825 or

286 NIC59 using PCR with degenerate primers (Figure S3A). Additionally, treatment of both strains

287 with tetracycline for nine generations failed to rescue hybrid inviability (Figure S3B).

288 Endosymbiotic bacteria within the *Rickettsiales* order are typically susceptible to tetracycline

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289 (McOrist 2000; Darby *et al.* 2015). Thus, endosymbiotic bacteria are unlikely to cause the

290 reproductive barrier between NIC59 and JU1825.

291

292 **Cytoplasmic-nuclear incompatibility is common within *C. nouraguensis***

293   We hybridized additional wild-isolates to determine whether cytoplasmic-nuclear

294 incompatibilities represent a common reproductive barrier within *C. nouraguensis,* or whether they

295 are an unusual phenotype only observed in hybridizations between NIC59 and JU1825.

296 Specifically, we tested the compatibility of four cytoplasmic genotypes with seven nuclear

297 genotypes (Figure 3). To test for an incompatibility between one strain's cytoplasm and another

298 strain's nuclear genome, we again compared the viabilities of backcrosses that differ only in the

299 F1 hybrid female's cytoplasmic genotype (Figure 3A). Specifically, we compared the viability of the

300 backcross that combines heterotypic cytoplasmic and nuclear genotypes to the viability of the

301 backcross that combines homotypic cytoplasmic and nuclear genotypes. We calculated the

302 relative viability of the two crosses (i.e. heterotypic combination / homotypic combination), and

303 tested for statistically significant differences (see Materials and Methods). Using the same logic as

304 for our JU1825 x NIC59 crosses, we reasoned that lower viability of the heterotypic cytoplasmic-

305 nuclear combination in comparison to the homotypic cytoplasmic-nuclear combination indicates a

306 cytoplasmic-nuclear incompatibility. Three or four biological replicates were performed for each

307 cytoplasmic-nuclear combination.

308   Of the 74 cytoplasmic-nuclear tests performed, 50 (67%) exhibited significant

309 incompatibilities (Figure 3B). Additionally, each cytoplasmic genotype was consistently

310 incompatible with at least one heterotypic nuclear genotype (i.e. all replicates for a particular

311 cytoplasmic-nuclear combination indicate a significant incompatibility). However, there are a

312 number of cytoplasmic-nuclear combinations whose replicates are inconsistent with one another

313 (e.g. some replicates indicate a significant incompatibility while others do not) (Figure 3C and

314 Figure S4). This may indicate that the genetic loci required for hybrid inviability are not fixed

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315   between the strains, but rather are polymorphisms segregating within each strain. This is

316   consistent with the fact that none of these strains have been formally inbred. Regardless, given

317   their common occurrence in hybridizations between strains of *C. nouraguensis,* we hypothesize

318   that cytoplasmic-nuclear incompatibilities are a significant reproductive barrier within the species.

319   We generated a heat map to help visualize the median relative viability for each

320   cytoplasmic-nuclear combination (Figure 3C). Strikingly, the NIC59 cytoplasmic genotype exhibits

321   a distinct response to hybridization, being strongly incompatible (i.e. having a low median relative

322   viability) with all of the nuclear genotypes tested. By comparison, the other cytoplasmic genotypes

323   can be relatively compatible with some heterotypic nuclear genotypes or exhibit incompatibilities

324   that are typically weaker than those involving the NIC59 cytoplasmic genotype. Specifically,

325   incompatibilities involving the JU1837 or JU1854 cytoplasmic genotypes have significantly higher

326   relative viability (median=0.72 and 0.71, respectively) in comparison to incompatibilities with the

327   NIC59 cytoplasmic genotype (median=0.45) (Figure 3B). Incompatibilities involving the JU1825

328   cytoplasm exhibit an intermediate level of relative viability (median=0.64) that is statistically

329   indistinguishable from the other cytoplasmic genotypes (P=0.057 in comparison to NIC59; P=1.0

330   in comparison to both JU1837 and JU1854). We conclude that the NIC59 cytoplasmic genotype is

331   distinct in terms of the nuclear genotypes it is incompatible with and how severe those

332   incompatibilities are.

333

334   **A single BDM incompatibility between a NIC59 cytoplasmic locus and a JU1825 nuclear**

335   **locus causes embryonic lethality**

336   As previously discussed, the backcross that combines the NIC59 cytoplasmic genotype

337   with JU1825 nuclear genotype (i.e. (N); N/J F1 female x JU1825 male, Figure 2B) results in only

338   ~36% of F2 offspring maturing to the L4 or young adult stage. A more detailed characterization of

339   F2 inviability shows that ~50% of F2 offspring fail to complete embryogenesis (Figure 4A). Of the

340   remaining half that complete embryogenesis, ~33% fail to mature to the L4 or young adult stage

341 (data not shown). In comparison, (J); N/J F1 female x JU1825 male crosses result in low levels of

342 embryonic lethality, similar to parental crosses. These data are consistent with F2 embryonic

343 lethality resulting from a single BDM incompatibility between a NIC59 cytoplasmic locus and a

344 single homozygous JU1825 autosomal locus.

345 To test the hypothesis of a single BDM incompatibility, we crossed viable F2 females to

346 JU1825 males and assayed F3 viability. Under this hypothesis, the surviving F2 females are

347 expected to have inherited NIC59 mtDNA and be heterozygous (i.e. JU1825/NIC59) at the

348 JU1825 nuclear incompatibility locus (Figure 6A). Therefore, crossing these F2 females to JU1825

349 males should also result in ~50% embryonic lethality in the F3 generation. This pattern should

350 also be true for additional backcross generations (e.g. F4, F5 etc.). Thus, we generated 15

351 independent backcross lineages, each consisting of matings between single surviving hybrid

352 females and JU1825 males, and monitored each lineage's viability for four backcross generations.

353 Indeed, the approximately 50% embryonic lethality observed in the F2 generation is also observed

354 in the subsequent backcross generations in all lineages (Figure 4B). These results are consistent

355 with the hypothesis that embryonic lethality is the result of a simple two-locus BDM incompatibility

356 between a purely maternally inherited cytoplasmic NIC59 locus and a single nuclear locus

357 homozygous for JU1825 alleles. We hypothesize that the post-embryonic inviability may be a

358 genetically separable phenotype.

359

360 **The JU1825 cytoplasm appears to be heteroplasmic**

361 As previously discussed, the backcross that combines the JU1825 cytoplasmic genotype

362 with the NIC59 nuclear genotype (i.e. (J); N/J F1 female x NIC59 male crosses) results in ~50%

363 F2 viability on average (Figure 2B). Thus, the total F2 inviability could be the result of a single

364 BDM incompatibility between a JU1825 cytoplasmic locus and a single autosomal locus

365 homozygous for NIC59 alleles.

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366   To test this hypothesis, we generated 15 independent backcross lineages, each consisting

367   of matings between single surviving hybrid females and NIC59 males, and monitored each

368   lineage's viability for four backcross generations. To our surprise, while some lineages continued

369   to exhibit low levels of viability similar to the F2 generation average (~50%), others began to

370   exhibit and maintain significantly increased viability for multiple backcross generations (Figure

371   5A). For example, in this particular experiment we found that in the F2 generation a majority of

372   lineages (13/15) had a total viability ranging from 18-50%, while only two exhibited higher viability

373   (68% and 85%). However, by the F5 backcross generation, we found that of the fourteen

374   remaining lineages only four exhibited 50% viability or less. Strikingly, by the F5 generation, 5/14

375   backcross lineages exhibited nearly 100% viability.

376   The rescue of hybrid inviability for some lineages via several generations of backcrossing is

377   peculiar. One hypothesis to explain this phenomenon is that the JU1825 cytoplasmic and/or

378   NIC59 nuclear incompatibility loci are not fixed within their respective strains, but rather are

379   segregating polymorphisms. As a specific example, the JU1825 cytoplasmic incompatibility locus

380   could be heteroplasmic for alleles that are either incompatible or compatible with the NIC59

381   nuclear genome. The mitochondrial genome is present at a high copy number within a single cell,

382   and it is thought that individual mtDNAs are randomly replicated and segregated to daughter cells

383   during cell division. Studies on the inheritance of various mtDNA heteroplasmies show that their

384   frequency amongst siblings from the same mother can be highly variable due to the random

385   sampling of mtDNAs and genetic bottlenecks during female germline development (Wallace and

386   Chalkia 2013). Therefore, it is possible that a NIC59-compatible cytoplasmic allele has increased

387   in frequency in some backcross lineages and rescued inviability.

388   To gain a better understanding of the genetic composition of the JU1825 cytoplasm, we

389   also monitored the viability of (J); N/J female x JU1825 male lineages over four backcross

390   generations. Because this cross combines homotypic JU1825 cytoplasmic and JU1825 nuclear

391   genotypes, we originally predicted that the relatively high rates of F2 viability would persist or

18

392 possibly increase with additional backcross generations. However, we instead observed that some

393 backcross lineages showed a striking decrease in viability after the F2 generation (Figure 5B). For

394 example, in this particular experiment, lineages in the F2 generation exhibited a uniform

395 distribution of viability, with an average of 74%. By the F5 generation we find two distinct

396 populations of lineages, those with a high viability ranging from 85-96% (6/14 lineages) and those

397 with an astonishingly low viability ranging from 29-55% (8/14 lineages) (Figure 5B). The latter

398 population has an average viability of 39%, which is quite similar to that observed in (N); N/J F1

399 female x JU1825 male crosses (~36%, Figure 2B), indicating that although these lineages

400 inherited their cytoplasm from JU1825 mothers, they now seem to exhibit low levels of viability

401 similar to those observed in the NIC59 cytoplasmic – JU1825 nuclear incompatibility. One

402 hypothesis to explain these data is that the JU1825 cytoplasm harbors a NIC59-like allele which at

403 a certain threshold frequency can mimic the NIC59 cytoplasmic-JU1825 nuclear incompatibility in

404 certain (J); N/J F1 female x JU1825 male backcross lineages.

405   In support of this hypothesis, the rate of embryonic lethality for some (J); N/J female x

406 JU1825 male backcross lineages also increases to levels observed in the NIC59 cytoplasmic –

407 JU1825 nuclear incompatibility (i.e. 50%) and can be stably inherited for several backcross

408 generations (Figure 5C). Specifically, most lineages (12/14) in the F2 generation exhibited only 0-

409 19% embryonic lethality, while only two lineages exhibited higher rates (38 and 47%). However,

410 by the F5 backcross generation, only about half of the lineages (6/14) exhibited 0-8% embryonic

411 lethality, while 8/14 lineages exhibited 35-65% embryonic lethality. Taken together, the results

412 from the two backcross experiments are consistent with the hypothesis that the JU1825 cytoplasm

413 is heteroplasmic and harbors both JU1825-like and NIC59-like incompatibility loci (Figure 6B and

414 C).

415

19

DISCUSSION

416   We discovered a lethal cytoplasmic-nuclear incompatibility between two wild isolates of *C. nouraguensis*, JU1825 and NIC59, and find that such incompatibilities may be widespread between other wild-isolates within the species. We show that maternally inherited endosymbiotic bacteria are not the cause of hybrid inviability, making the mitochondrial genome the most likely candidate for harboring the cytoplasmic incompatibility factor(s). We also propose that the JU1825 cytoplasm is heteroplasmic, and harbors both JU1825-like and NIC59-like incompatibility loci.

423   In eukaryotes, the mitochondrial genome typically contains a very small fraction of the gene content of a cell, yet it seems to be involved in a disproportionate number of genetic incompatibilities across a diverse range of taxa (Rand *et al.* 2004; Burton and Barreto 2012) . However, there are relatively few cases in which incompatibility loci have been definitively mapped to the mitochondrial genome, and therefore a larger sample is required to better understand what drives the evolution of mitochondrial-nuclear incompatibility. Additionally, all of the molecularly identified cases of mitochondrial-nuclear incompatibility have been found between species rather than within species (Lee *et al.* 2008; Chou *et al.* 2010; Luo *et al.* 2013; Meiklejohn *et al.* 2013; Ma *et al.* 2016). Some of these inter-species hybridizations harbor additional genetic incompatibilities or chromosomal rearrangements that cause inviability and sterility (Hunter *et al.* 1996; Fischer *et al.* 2000; Brideau *et al.* 2006; Ferree and Barbash 2009; Mihola *et al.* 2009; Davies *et al.* 2016), making it difficult to discern whether mitochondrial-nuclear incompatibility was instrumental in initiating speciation or evolved after strong reproductive isolation occurred. The incompatibility we describe here provides an excellent opportunity to study the evolutionary genetics and cell biology of incipient speciation as well as mitochondrial-nuclear incompatibility. The ease of breeding, large brood sizes, and short generation time of *C. nouraguensis* should facilitate the mapping and identification of the genes that contribute to hybrid inviability.

Asymmetric cytoplasmic-nuclear incompatibilities

20

442   Most molecularly characterized BDM incompatibilities are asymmetric, in that only one of

443   two divergent alleles at a locus is incompatible with heterospecific alleles at other loci (e.g.

444   Brideau *et al.* 2006; Ferree and Barbash 2009). This is also true of the mitochondrial-nuclear

445   incompatibilities seen in *Saccharomyces* species hybridizations (Lee *et al.* 2008; Chou *et al.*

446   2010). For example, an intron of the *COX-1* gene in the *Saccharomyces bayanus* mitochondrial

447   genome fails to be correctly spliced by the nuclearly encoded *S. cerevisiae MRS-1* gene, resulting

448   in hybrid inviability presumably due to a failure of respiration on non-fermentable media. However,

449   a similar incompatibility does not occur between *S. cerevisiae COX-1* and *S. bayanus MRS-1.* At

450   first glance it may seem as though the cytoplasmic-nuclear incompatibility between JU1825 and

451   NIC59 is symmetric, since both strains have a cytoplasm that is incompatible with the other

452   strain's nuclear genome. However we currently cannot determine whether the JU1825 or NIC59

453   cytoplasmic and nuclear incompatibility loci are allelic, leaving open the possibility that different

454   genes cause hybrid inviability in the reciprocal crosses.

455

456   **Cytoplasmic-nuclear incompatibility: both sexes are equally inviable**

457   J.B.S Haldane noted that the heterogametic sex more often suffers from inviability or

458   sterility in inter-species hybridizations than the homogametic sex (Delph and Demuth 2016). It is

459   not known whether "Haldane's rule" also applies in intra-species hybridizations. The lethal

460   cytoplasmic-nuclear incompatibility we identified between the NIC59 and JU1825 wild isolates of

461   *C. nouraguensis* affects females and males equally, suggesting that the two sexes share the

462   same disrupted developmental pathway(s). However, we have not carefully studied other aspects

463   of sex-specific fitness, such as female and male F2 hybrid fertility. Because the mitochondrial

464   genome is inherited only through females, theory predicts that evolution will lead to the

465   accumulation of mtDNA variants that are neutral or increase female fitness, but that are neutral or

466   possibly deleterious to male fitness (Gemmell *et al.* 2004). Because of this, male-specific functions

467   may be more adversely affected during the hybridization of heterotypic mitochondrial and nuclear

21

468   genomes. This is indeed the case for some known mitochondrial-nuclear incompatibilities. For

469   example, when swapping the mitochondrial genomes between mouse subspecies via pronuclear

470   transfer, one mitochondrial-nuclear combination resulted in reduced male fertility while females

471   had relatively normal fertility (Ma *et al.* 2016). Therefore, further studies of *C. nouraguensis* hybrid

472   male fertility are required to more fully address whether this system follows Haldane's rule, as well

473   as to determine whether there are male-specific mitochondrial-nuclear incompatibilities.

474

475   **JU1825 heteroplasmy**

476   We hypothesize that the JU1825 cytoplasm is heteroplasmic and contains mitochondrial

477   genomes that are both compatible (JU1825-like) and incompatible (NIC59-like) with the JU1825

478   nuclear incompatibility locus. If the JU1825 cytoplasm is naturally heteroplasmic, we predict the

479   NIC59-like mtDNAs are kept at a low frequency within JU1825 by selection. This selection would

480   be relaxed in (J); N/J F1 hybrids and the frequency of NIC59-like mtDNA can increase, reducing

481   incompatibility in backcrosses to NIC59 males and increasing incompatibility in backcrosses to

482   JU1825 males. However, our data cannot rule out the possibility that NIC59-like mtDNA is

483   introduced into F1 females by incomplete degradation and inheritance of paternal NIC59 mtDNA.

484   Interestingly, indirect evidence suggests that paternal mtDNA can be inherited when hybridizing

485   different wild isolates of *Caenorhabditis briggsae* (Hicks *et al.* 2012; Chang *et al.* 2015).

486   The hypothesized heteroplasmy of the JU1825 cytoplasm may explain the greater variance

487   of F2 viability in crosses with (J); N/J F1 females in comparison to those with presumably

488   homoplasmic (N); N/J F1 females. Stochastic segregation and genetic bottlenecking events from

489   JU1825 mothers may result in F1 females with a wide range of frequencies of the NIC59-like

490   cytoplasmic allele, and therefore a wide range of F2 viability when backcrossed to either NIC59 or

491   JU1825 males. This stochastic inheritance may also explain why the degree of F2 viability of (J);

492   N/J F1 female x JU1825 male backcrosses can also vary significantly from experiment to

493   experiment (Figure S1).

22

494

Acknowledgments

502

503 **Figure Legends**

504 **Figure 1. JU1825 and NIC59 exhibit F2 hybrid breakdown**. Crosses are listed on the y-axis.

505 Letters in parentheses to the left of a semi-colon denote the cytoplasmic genotype of an individual

506 (e.g. "(J)" individuals have a JU1825 cytoplasmic genotype), while letters to the right of a semi-

507 colon denote the genotypes of all autosomal loci (i.e. "N/J" individuals are heterozygous

508 NIC59/JU1825 throughout the autosomes). Only (J); N/J F1 x (J); N/J F1 and (N); N/J F1 x (N);

509 N/J F1 crosses exhibit a significant decrease in the percentage of viable progeny (P<0.01 and

510 P<0.001, respectively). There are no significant differences in the percentages of viable males

511 between crosses (P>0.05). N=14 or 15 plates per cross. All p-values were calculated by a

512 Kruskal-Wallis test followed by Dunn's test.

513

514 **Figure 2. F2 inviability involves a maternal cytoplasmic effect. (A)** There is no significant

515 difference in the percentage of viable progeny between any of the F1 hybrid male backcrosses

516 and intra-strain crosses (P>0.05). **(B)** Backcrossing hybrid females to parental strain males

517 reveals that only (N); N/J F1 female x JU1825 male crosses and (J); N/J F1 female x NIC59 male

518 crosses exhibit a significant decrease in the percentage of viable progeny in comparison to intra-

519 strain crosses (P<0.001). (N); N/J F1 female x JU1825 male crosses have significantly decreased

520 viability in comparison to (J); N/J F1 female x JU1825 male crosses (P<0.001). Additionally, (J);

521 N/J F1 female x NIC59 male crosses consistently have significantly decreased viability in

522 comparison to (N); N/J F1 female x NIC59 male crosses (P<0.05). The viability of (J); N/J F1

523 female x JU1825 males can differ significantly between experiments (Figure S1). There are no

524 significant differences in the proportion of viable males between the crosses (P>0.05). N=14 or 15

525 plates per cross. All p-values were calculated by a Kruskal-Wallis test followed by Dunn's test.

526

527 **Figure 3. Cytoplasmic nuclear incompatibility is widespread within *C. nouraguensis*. (A)**

24

528 To determine whether a particular cytoplasmic-nuclear combination is incompatible, we tested for

529 statistical differences in viability between the F1 female backcross that combines heterotypic

530 cytoplasmic and nuclear genotypes (top cross) and the backcross that combines homotypic

531 cytoplasmic and nuclear genotypes (bottom cross, see Materials and Methods). We also

532 calculated the relative viability of the first cross to the second. **(B)** A scatter plot depicting all the

533 cytoplasmic-nuclear compatibility tests performed. Each point corresponds to a single replicate of

534 a certain cytoplasmic-nuclear combination. Points above the horizontal dashed gray line indicate

535 statistically significant differences in viability between the two types of crosses mentioned in (A)

536 (P<0.0006 after Bonferroni correction, Fisher's exact test). Points above the horizontal dashed

537 gray line that have a relative viability <1 are considered statistically significant cytoplasmic-nuclear

538 incompatibilities. The color of a point corresponds to the cytoplasmic genotype being tested. All

539 cytoplasmic genotypes tested show an incompatibility with one or more heterotypic nuclear

540 genotypes. See Figure S4 for separate graphs of all combinations. Above the scatterplot are

541 boxplots depicting the relative viabilities of statistically significant cytoplasmic-nuclear

542 incompatibilities. The color corresponds to cytoplasmic genotype tested. Incompatibilities involving

543 the NIC59 cytoplasmic genotype have reduced viability compared to those involving the JU1837

544 and JU1854 cytoplasmic genotypes (P<0.001, Kruskal-Wallis test followed by Dunn's test). **(C)** A

545 heatmap depicting the median relative viability for each cytoplasmic-nuclear combination. Each

546 cytoplasmic-nuclear combination shows the proportion of replicates that exhibit significant

547 incompatibilities (e.g. 3 out of 3 replicates exhibit significant incompatibilities for the NIC59

548 cytoplasm – JU1854 nuclear combination, while only 1 out of 3 replicates exhibit significant

549 incompatibilities for the JU1837 cytoplasm – JU1854 nuclear combination). Each cytoplasmic

550 genotype is consistently incompatible with at least one heterotypic nuclear genotype. The NIC59

551 cytoplasm has a distinct response to hybridization than the others tested.

552

25

553   **Figure 4. A single BDM incompatibility between a NIC59 cytoplasmic locus and a JU1825**

554   **nuclear locus causes embryonic lethality. (A)** Approximately 50% of the F2 progeny from (N);

555   N/J F1 female x JU1825 male crosses arrest during embryogenesis, significantly higher than that

556   seen in intra-strain crosses (P<0.001). In contrast, (J); N/J F1 female x JU1825 male and parental

557   strain crosses exhibit low and similar levels of embryonic lethality (P>0.05). **(B)** Initially, fifteen (N);

558   N/J F1 females were independently backcrossed to single JU1825 males. For each independent

559   lineage, a single surviving F2 female was again backcrossed to a JU1825 male. This

560   backcrossing scheme was repeated until the F5 generation (11 lineages remain). Each colored

561   line represents a single backcross lineage. All backcross lineages exhibit ~50% embryonic

562   lethality throughout the backcross generations, consistent with the hypothesis that an

563   incompatibility between a NIC59 cytoplasmic locus and a single JU1825 nuclear locus causes

564   embryonic lethality. **(C)** Both parental strains were "backcrossed" as a negative control (JU1825

565   =14 backcross lineages in the F1 generation, 11 lineages by the F4 generation; NIC59 = 15

566   backcross lineages in the F1 generation, 11 lineages by the F4 generation). All p-values were

567   calculated by a Kruskal-Wallis test followed by Dunn's test.

568

569   **Figure 5. The JU1825 cytoplasm is heteroplasmic for JU1825-like and NIC59-like alleles. (A)**

570   The viability of 15 independent (J); N/J female x NIC59 male backcross lineages were followed

571   until the F5 generation. Surprisingly, in some lineages, multiple generations of backcrossing

572   resulted in increased viability (similar to that seen in intra-strain crosses). **(B)** The viability of 15

573   independent (J); N/J female x JU1825 male backcross lineages were also followed until the F5

574   generation. Interestingly, multiple generations of backcrossing results in some lineages with

575   significantly reduced viability, similar to that seen in (N); N/J F1 female x JU1825 male crosses.

576   We also examined embryonic lethality, finding that some lineages exhibit the ~50% embryonic

577   lethality seen in (N); N/J F1 female x JU1825 male crosses upon additional generations of

26

578   backcrossing. These results are consistent with the hypothesis that the JU1825 cytoplasm is

579   heteroplasmic and contains JU1825-like and NIC59-like alleles.

580

581   **Figure 6. Mitochondrial-nuclear incompatibility model. (A)** We hypothesize that F2 hybrid

582   breakdown is the result of a Bateson-Dobzhansky-Muller incompatibility between the NIC59

583   mitochondrial genome and a nuclear locus homozygous for the JU1825 allele, and vice versa. As

584   a specific example, when NIC59 females are crossed to JU1825 males, the resulting F1 hybrid

585   females are expected to be heterozygous at all autosomal loci while inheriting only NIC59 mtDNA.

586   When F1 females are backcrossed to JU1825 males, F2 inviability results from an incompatibility

587   between NIC59 mtDNA and an autosomal locus homozygous for the JU1825 nuclear allele. **(B)**

588   We hypothesize that the JU1825 cytoplasm is heteroplasmic in F1 females and contains at least

589   one NIC59-like allele. Backcrossing hybrid females with a JU1825 cytoplasm (i.e. (J); N/J females)

590   to NIC59 males for multiple generations can allow the NIC59-like cytoplasmic allele to increase in

591   frequency and dilute out the effects of the incompatible JU1825-like mtDNA (e.g. top right F2

592   female). This eventually may allow the NIC59 nuclear locus to become homozygous and restore

593   the viability of a lineage. On the other hand, the NIC59-like mtDNA can stay at a low frequency in

594   viable F2 females (e.g. bottom right F2 female). Backcrossing these F2 females to NIC59 males

595   results in levels of inviability similar to the F2 generation. **(C)** By a similar line of reasoning,

596   backcrossing hybrid females with a JU1825 cytoplasm to JU1825 males for multiple generations

597   can allow the NIC59-like mtDNA to increase in frequency, where it can mimic the same genetic

598   incompatibility seen in (N); N/J F1 female x JU1825 male crosses (Figure 6A).

599

600   **Supplemental Figure 1. Variability of (J); N/J F1 female x JU1825 male crosses across**

601   **experiments.** Three biological replicates of the same type of backcross experiment. (J); N/J F1

602   female x JU1825 male crosses can either exhibit similar or significantly decreased rates of viability

603   in comparison to intra-strain crosses across experiments (Experiment 1, non-significant, $P > 0.05$;

604   Experiment 2, non-significant, P>0.05; Experiment 3, P>0.05, non-significant in comparison to

605   JU1825 x JU1825 crosses, P<0.05 significant in comparison to NIC59 x NIC59 crosses).

606   However, (J); N/J F1 female x JU1825 male crosses consistently exhibit significantly increased

607   rates of viability in comparison to (N); N/J F1 female x JU1825 male crosses (Experiment 1, **,

608   P<0.01; Experiment 2, **, P<0.01; Experiment 3, *, P<0.05). Experiments 1 and 2 are data from

609   Figures 2 and 5, respectively. All p-values were calculated by a Kruskal-Wallis test followed by

610   Dunn's test.

611

612   **Supplemental Figure 2. Nuclear incompatibility loci are linked to autosomes, not sex**

613   **chromosomes.** F1 intercrosses allow us to infer that the nuclear incompatibility loci are

614   autosomal, not X-linked. From the (N); N/J F1 x JU1825 male backcross experiment (Figure 2),

615   we concluded that F2 inviability was the result of a genetic incompatibility between the NIC59

616   mitochondrial genome and nuclear loci homozygous or hemizygous for JU1825 alleles. It is

617   reasonable to assume that the same genetic incompatibility contributes to F2 inviability in (N); N/J

618   F1 female x (N); N/J F1 male crosses. If the nuclear incompatibility locus were X-linked, F2 males

619   would have a 50% chance of being hemizygous for the JU1825 nuclear incompatibility locus while

620   F2 females can only be heterozygous or homozygous for NIC59 alleles. Therefore, if the locus

621   were X-linked, half of F2 males would be inviable while females would be unaffected. If the

622   nuclear incompatibility locus were autosomally linked, then both sexes have an equal chance of

623   being homozygous for the JU1825 nuclear incompatibility locus. Therefore, both sexes are

624   expected to suffer equal rates of inviability. We do not observe a significant decrease in the

625   proportion of viable F2 males (Figure 1), so we conclude that the JU1825 nuclear incompatibility

626   locus or loci are linked to autosomes. The same line of reasoning can be used to show that the

627   NIC59 incompatibility locus or loci are also autosomally linked.

628

629 **Supplemental Figure 3. Endosymbiotic bacteria do not cause cytoplasmic-nuclear**

630 **incompatibility. (A)** PCR on both JU1825 and NIC59 crude lysates (10 adult worms per lysate, 5

631 females and 5 males) with degenerate primers against the *Wolbachia fbpA* or *gatB* loci fails to

632 amplify the expected products. w[1118] (wol+) and w[1118] (wol-) *D. melanogaster* flies serve as

633 positive and negative controls, respectively. PCR on crude lysates of OP50 (bacterial food source

634 of NIC59 and JU1825) also fails to amplify the expected products. PCR on JU2079, an inbred

635 strain derived from JU1825, also fails to amplify the expected *gatB* product. **(B)** Both tetracycline-

636 treated (J);N/J F1 female x NIC59 male and (N);N/J F1 female x JU1825 male crosses exhibit

637 significantly decreased levels of viability in comparison to tetracycline treated intra-strain crosses

638 (P<0.01). Additionally, there are no statistical differences in viability between NIC59 x NIC59 and

639 JU1825 x JU1825 tetracycline treated intra-strain crosses (P>0.05). N=14 or 15 for each cross. All

640 p-values were calculated by a Kruskal-Wallis test followed by Dunn's test.

641

642 **Supplemental Figure 4. Cytoplasmic-nuclear tests separated by nuclear genotype.** Each

643 graph depicts all the cytoplasmic-nuclear tests performed between four cytoplasmic genotypes

644 and a single nuclear genotype. This is the same data that is grouped into a single graph in Figure

645 3B. Each cytoplasmic-nuclear combination has three biological replicates (except for JU1825

646 cytoplasm – NIC24 nuclear and JU1825 cytoplasm – NIC54 nuclear combinations, which have

647 four replicates). Although there appear to be many cases of significant cytoplasmic-nuclear

648 incompatibility (relative viability<1 and P<0.0006 after Bonferroni correction), there can be

649 discrepancies between replicates (e.g. one replicate of the JU1825 cytoplasm – NIC24 nuclear

650 combination indicates a significant incompatibility, while the other three do not).

651

29

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791

Figure 1

A
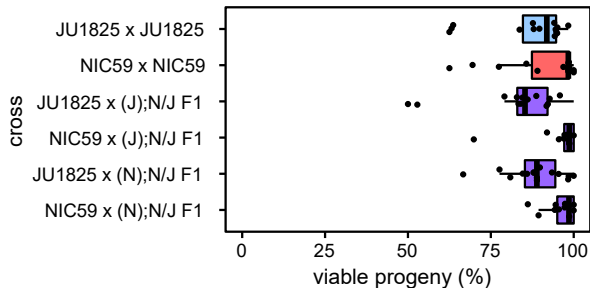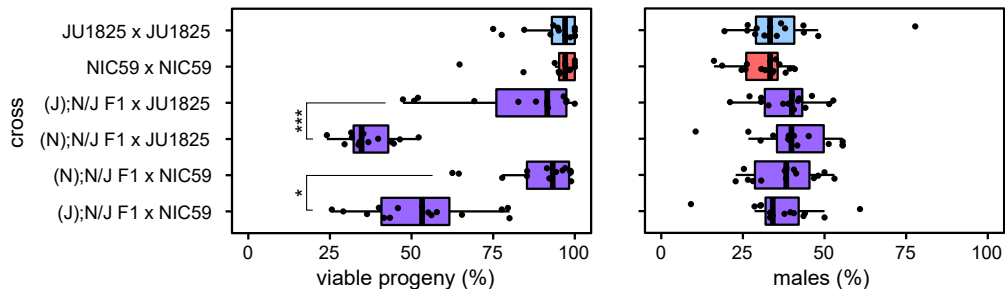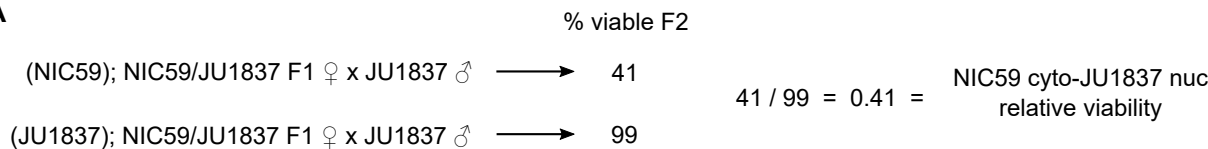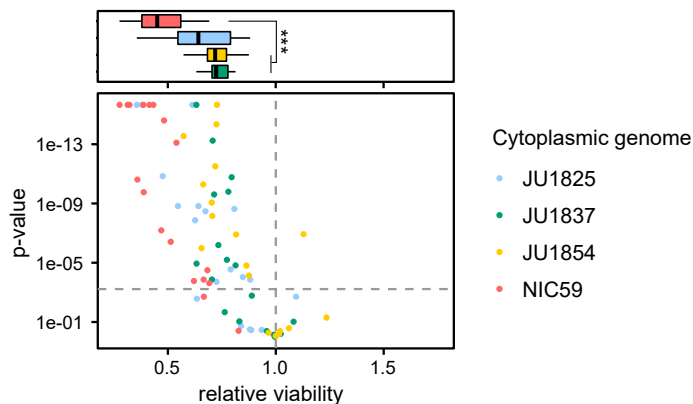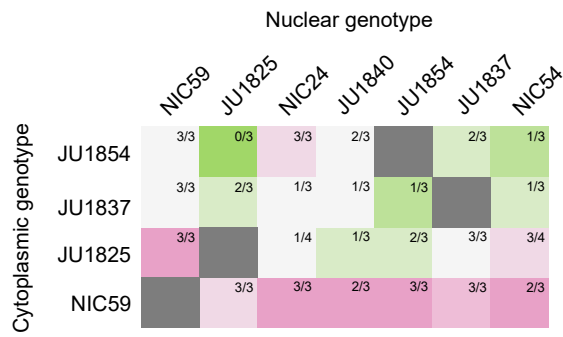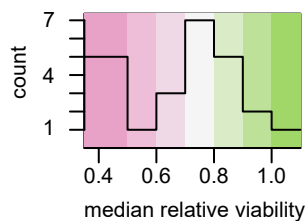
Figure 2

Figure 3

Figure 4

Figure 5

A (J);N/J x NIC59

B (J);N/J x JU1825

(J);N/J x JU1825

Figure 6

A

B

C