1   Title: The ebb and flow of heteroplasmy during intra-species hybridization in *Caenorhabditis*

2   *briggsae*

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4   Authors:

5   Shadi Adineh

6   Joseph A. Ross

7

8   Affiliation:

9   Department of Biology

10   California State University, Fresno

11   Fresno, CA 93740

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18   Contact information for Corresponding Author:

19   Dr. Joseph A. Ross

20

21   jross@csufresno.edu

22

23   +1-559-278-4074

24

25   Department of Biology

26   2555 E. San Ramon Ave

27   M/S SB73

28   Fresno, CA 93740

29

30   ABSTRACT

31   Mitochondria are typically maternally inherited. In many species, this transmission

32   pattern is produced by sperm-borne mitochondria being eliminated either from sperm before

33   fertilization or from the embryo after fertilization. In the nematode *Caenorhabditis briggsae*,

34   repeatedly backcrossing hybrids to genetically diverse males can elicit paternal mitochondrial

35   transmission. Studies of other taxa also suggest that hybridization increases paternal

36   mitochondrial transmission. Thus, hybrid genotypes might disrupt the systems that normally

37   prevent paternal mitochondrial transmission. Given the reliance of a number of genetic analyses

38   on the assumption of purely maternal mitochondrial inheritance, it would be broadly valuable to

39   learn more about the processes embryos employ to prevent sperm-borne mitochondria from

40   persisting in offspring, as well as the circumstances under which paternal transmission might be

41   expected to occur. To quantify the tempo of paternal mitochondrial transmission in hybrids, we

42   assessed the presence of paternal mitotypes in replicate lines at three timepoints spanning fifteen

43   generations. All lines exhibited paternal mitochondrial transmission. However, this heteroplasmy

44   always then resolved to homoplasmy for the maternal mitotype. Additionally, one nuclear locus

45   exhibited allele transmission ratio distortion that might reflect mito-nuclear co-evolution. This

46   study frames the genetic architecture of a hybrid genetic incompatibility that leads to paternal

47   mitochondrial transmission and to a reduction in hybrid fitness.

48

49   INTRODUCTION

50   Mitochondria play a pivotal role in cellular metabolism (Taylor and Turnbull 2005).

51   Much of the mitochondrial genome (mtDNA) encodes genes essential for transcription and

52   translation as well as the oxidative phosphorylation system (Bereiter-Hahn and Voth 1994;

53   Timmis, et al. 2004). Nuclear genes also encode protein subunits of electron transport chain

54   complexes. The reliance of mitochondrial function on genes encoded by two genomes

55   established an evolutionary dynamic where mitochondrial and nuclear genes co-evolve (Blier, et

56   al. 2001). Deleterious mutations in the mitochondrial genome can be compensated by mutations

57   in nuclear genes encoding adjacent protein subunits (Rand, et al. 2004). When hybridization

58   occurs between lineages carrying different co-evolved genomes, hybrid breakdown can result

59   from separation of co-evolved genes and subsequent mitochondrial dysfunction (Gershoni, et al.

60   2009).

61   Eukaryotes typically exhibit inheritance of mtDNA from their mothers (uniparental

62   inheritance) (Gyllensten, et al. 1991; Hurst 1992; Fontaine, et al. 2007), although empirical data

63   have occasionally shown the presence of both paternal and maternal mtDNA haplotypes

64   (mitotypes) in one individual (e.g. (Birky 1995)). In some species, like mussels, uniparental

65   inheritance does not occur (Zouros, et al. 1992; Zouros, et al. 1994). The existence of

66   predominantly (if not exclusively) maternal mtDNA inheritance might prevent inheritance of

67   sperm mtDNA that carry deleterious mutations (Mazat, et al. 2001). The presence of two

68   mitotypes (paternal and maternal) within a single zygote (termed heteroplasmy) might also be

69   unfavorable (Hurst 1992; Sharpley, et al. 2012) (Sato and Sato 2013), but see also (Ye, et al.

70   2014). Thus, maternal mitochondrial inheritance might exist to ensure homoplasmy, the presence

71   of a single mitochondrial haplotype in a cell.

72   At least two paternal mitochondrial elimination mechanisms exist: mitochondria in sperm

73   can be destroyed prior to fertilization, and mitochondria that enter the oocyte can be eliminated

74   after fertilization. For example, mammalian sperm enter the oocyte at fertilization, and their

75   mitochondria are tagged with ubiquitin for destruction by the proteasome (Sutovsky, et al. 1999);

76   mouse mtDNA are also eliminated prior to fertilization (Luo, et al. 2013). In the nematode *C.*

77   *elegans*, evidence suggests roles for ubiquitin and mitophagy in removing paternally-transmitted

78   mitochondrial following fertilization (Al Rawi, et al. 2011; Sato and Sato 2011; Zhou, et al.

79   2016). Such evidence of active elimination of paternal mitochondria in the embryo suggests that

80   a molecular mechanism(s) in the oocyte detects some signal identifying sperm-borne

81   mitochondria.

82   Here, we have investigated the dynamics of paternal mitochondrial transmission (PMT)

83   in intra-specific *C. briggsae* hybrids, which are known to sustain occasional PMT (Chang, et al.

84   2016; Ross, et al. 2016). We created reciprocal sets of triplicate cytoplasmic-nuclear hybrid

85   (cybrid) lines initiated by mating the genetically distinct *C. briggsae* wild isolate strains AF16

86   and HK104, which are about one percent divergent in the nuclear genome (Hillier, et al. 2007).

87   These cybrid lines experimentally combine the nuclear genome of one strain with the

88   mitochondrial genome of the other. We genotyped individuals at various generations during line

89   production to assess the presence of paternal mitochondria and found that all lines exhibited

90   PMT during line production, but heteroplasmy eventually resolved in favor of homoplasmy for

91   the maternal mitotype in all lines. By genotyping genetic markers in the nuclear genome, we also

92 discovered a pattern of mito-nuclear linkage disequilibrium that implies the presence of co-

93 evolved loci. Together, these results support the interpretation that an inter-genomic genetic

94 incompatibility precipitates PMT in hybrids.

95

96   MATERIALS AND METHODS

97   AF16 and HK104 wild isolates were obtained from the Caenorhabditis Genetics Center,

98 which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). To best

99 control for the effect of hybridization in this experiment, the reciprocal control lines (FV83–5,

100 FV62–4) and the cybrid lines (FV59–61, FV86–8) were all initiated from the F1 hybrid

101 generation, with serial P0 paternal backcrosses then used to create cybrid lines and maternal

102 backcrosses used to create control lines (Figure 1). Strain husbandry and cybrid crosses were

103 conducted as previously described (Chang, et al. 2016).

104   Primer sequences for amplified fragment length polymorphism (AFLP) genotyping of

105 nuclear loci (II: cb-m26, III: cb-m205, IV: cb-m172 and cb-m67, V: cb-m103, X: cb-m124 and

106 cb-m127) and for restriction fragment length polymorphism (RFLP) genotyping of mtDNA loci

107 (cb18178, cb34440) were obtained from (Koboldt, et al. 2010).

108   Single-worm PCR involved isolating an individual adult hermaphrodite nematode in 5 µL

109 of single-worm lysis buffer (50 mM KCl, 10 mM TRIS pH 8.3, 2.5 mM MgCl2, 0.45% nonidet

110 P40, 0.45% tween-20, 0.01% gelatin, 1 mg/mL proteinase K). After snap-freezing at -80°C, each

111 worm was incubated at 60°C for 60 m. and then at 95°C for 15 m. 1 µL of worm lysate was then

112 used as template DNA for AFLP and RFLP PCR and analysis as described (Chang, et al. 2016).

113   The nematode lines will be made available to qualified researchers upon request.

114

115   RESULTS AND DISCUSSION

116   We speculated that negative mito-nuclear epistatic interactions in cybrids might elicit

117 PMT in the experimental lines. The presence of a paternal mitotype in any hybrid generation

118 would reveal PMT, and we expected that nuclear allele frequencies would not deviate from the

119 values expected for selectively neutral marker loci. We genotyped eight individuals from each of

120 the twelve lines at two nuclear loci and one mitochondrial locus. Limited locus sampling was

121 necessary given the small amount of genomic DNA obtained from a single worm.

122   The HK104 allele frequencies of each line at the B5, B10 and S5 generations at two

123   nuclear loci and one in the mtDNA are plotted in Figure 2. Each data point represents the

124   frequency calculated from the successful genotyping of between two to eight individuals. Panels

125   A and B of Figure 2 display the change in the frequency of the HK104 alleles at nuclear loci and

126   in the mtDNA in reciprocal crosses designed to create cybrids. The expected HK104 nuclear

127   allele fraction values at each generation (solid line, open diamond) are based on the neutral

128   expectation of allele inheritance. HK104 allele fraction values were quite variable at the B5 and

129   B10 generations. Some of this variation is likely due to the small number of individuals (between

130   two and eight) successfully genotyped at these generations; this variation in replication was

131   caused by the vagaries of success of single-worm genotyping by PCR. The expected HK104

132   mitochondrial allele fraction values at each generation (solid line, open circle) are based on the

133   assumption of strictly maternal inheritance (Figure 2). Thus, in AF16xHK104 crosses, at every

134   generation, we expected that all individuals would contain only HK104 mtDNA (HK104 allele

135   fraction = 1, Figure 2A). In the reciprocal cross, all individuals should only contain AF16

136   mitotypes (HK104 allele fraction = 0, Figure 2B). If, at any point, the observed mitochondrial

137   allele fraction value deviates from 1 or 0, respectively, then that would indicate that at least one

138   individual genotyped in that generation contained paternal mtDNA. In the B5 and B10

139   generations of both cybrid crosses, we did observe paternal mitotypes. For example, in the B10

140   generation of the AF16xHK104 cybrid cross (Figure 2A), four of eight individuals in strain

141   FV59 had paternal mitotypes; five of eight FV60 individuals had paternal mitotypes (Figure 2B).

142   Interestingly, we also observed evidence of paternal mitotypes in the B5 and B10 generations in

143   the control crosses (Figures 2C, D).

144   Once each line reached the fifth generation of serial selfing (S5), at which homozygosity

145   throughout the nuclear genome was expected (Figure 1), genomic DNA from populations of

146   >10,000 individuals was extracted from all twelve lines. Each sample was genotyped at seven

147   loci on four nuclear chromosomes and two on the mtDNA to confirm the expected genotypes.

148   On chromosomes II, V and X, the genotypes agreed with expectations based on the cross design:

149   all lines contained only paternal alleles at the loci we genotyped (Figure 3). In these lines,

150   mitochondrial genotypes also concurred at this timepoint: maternal mtDNA were exclusively

151   detected (Figure 3). Only chromosome IV loci demonstrated a different allele retention pattern.

152   At a nuclear locus, each line has an average 97% probability of retaining only paternal alleles

153   after five generations of selfing under the neutral expectation. However, in both triplicate sets of

154   cybrids, one of two chromosome IV loci (cb-m172) exclusively retained the P0 maternal allele,

155   while the six control lines exclusively retained the P0 paternal allele (Figure 3). At a second

156   chromosome IV locus (cb-m67), the paternal allele was retained in all three triplicate cybrids for

157   one cross direction and not the other (Figure 3). This bias toward retention of maternal nuclear

158   alleles is consistent with the possibility that a chromosome IV allele more tightly linked to cb-

159   m172 than to cb-m67 has co-evolved with the mtDNA. Such an epistatic interaction might

160   ensure that the only individuals surviving line creation were those that inherited the HK104 cb-

161   m172 allele with the HK104 mitotype and those that inherited the AF16 cb-m172 allele with the

162   AF16 mitotype. This interpretation of positive mito-nuclear epistasis is supported by evidence of

163   mito-nuclear co-evolution between chromosome IV alleles and mitotype in AF16-HK104

164   advanced-intercross recombinant inbred lines (Ross, et al. 2011).

165   Attempts to quantify PMT frequencies in natural populations have, in at least one case,

166   revealed no paternal mitotypes in offspring (Wolff, et al. 2008). In *Drosophila melanogaster*,

167   heteroplasmy is common in nature and can take many generations to resolve to homoplasmy

168   (Nunes, et al. 2013). However, in hybrids, studies from multiple taxa suggest that PMT increases

169   in frequency. For example, a study of the bird *Parus major* showed that introgression of a

170   paternal mitochondrial haplotype occurred following intra-species hybridization and generated a

171   single heteroplasmic individual (Kvist, et al. 2003). Heterospecific and conspecific hybridization

172   in mice, fruit flies, chickens, and other nematodes have also produced evidence of PMT (Shitara,

173   et al. 1998; Sherengul, et al. 2006; Hoolahan, et al. 2011; DeLuca and O'Farrell 2012; Wolff, et

174   al. 2013; Alexander, et al. 2015). Thus, a growing body of empirical evidence suggests that

175   hybridization increases the frequency of PMT. We favor the interpretation that hybridization

176   might separate co-evolved alleles involved in maternal detection and elimination of sperm-

177   transmitted mitochondria.

178   The current study adds to prior evidence of PMT in intra-species *C. briggsae* cybrids

179   (Chang, et al. 2016; Ross, et al. 2016). However, other AF16-HK104 hybrids, like a panel of 167

180   advanced-intercross recombinant inbred lines, do not contain paternal mitotypes (Ross, et al.

181   2011). This difference suggests a genetic architecture responsible for PMT that is specific to

182   genotypes generated by paternal backcrossing (e.g. mito-nuclear epistasis) and not related simply

183   to having a particular hybrid nuclear genotype. The possibility of a complex genetic basis of

184   PMT has been noted (Dokianakis and Ladoukakis 2014). It is likely that our findings are not

185   universal, because backcrosses in mice have revealed a lesser extent of PMT (Gyllensten, et al.

186   1991). Paternal backcross designs in other taxa have found no evidence of PMT (Lansman, et al.

187   1983; Avise and Vrijenhoek 1987), although it has been suggested that, in Drosophila, such

188   repeated hybridization is important for PMT (Wolff, et al. 2013).

189   We observed paternal mitotypes following hybridization in all lines. Why might we have

190   observed paternal mitotypes even in our control strains? It is possible that, because our

191   experimental and control strains were all initiated from F1 hybrids, they all had an opportunity to

192   experience PMT before the cross designs diverged to P0 paternal backcrosses (to generate

193   experimental cybrid lines) and P0 maternal backcrosses (to generate control lines).

194   Despite low replication of genotype collection in the B5 and B10 generations, three

195   salient data trends are evident. First, in each of the twelve lines, paternal mitotypes were always

196   detected in at least one individual. Second, the S5 generation lines all revealed consistent and

197   homozygous genotypes at each locus across all three replicates. Third, only loci on chromosome

198   IV at S5 ever contradicted the expected genotype based on the cross design. In sum, the evidence

199   of PMT into offspring following hybridization in an intra-species cross supports previous

200   observations in *C. briggsae* (Chang, et al. 2016; Ross, et al. 2016) as well as in other taxa.

201   Further, the nuclear allele retention pattern observed on chromosome IV might be evidence of

202   mito-nuclear epistasis that, when disrupted at hybridization, causes fitness reduction. Future

203   studies of this *C. briggsae* hybrid system will be valuable to elucidate the genetic interactions

204   that facilitate the elimination of paternal mitotypes at fertilization and further challenge the

205   dogma of strictly maternal mitochondrial inheritance (Carelli 2015).

206

207   ACKNOWLEDGMENTS
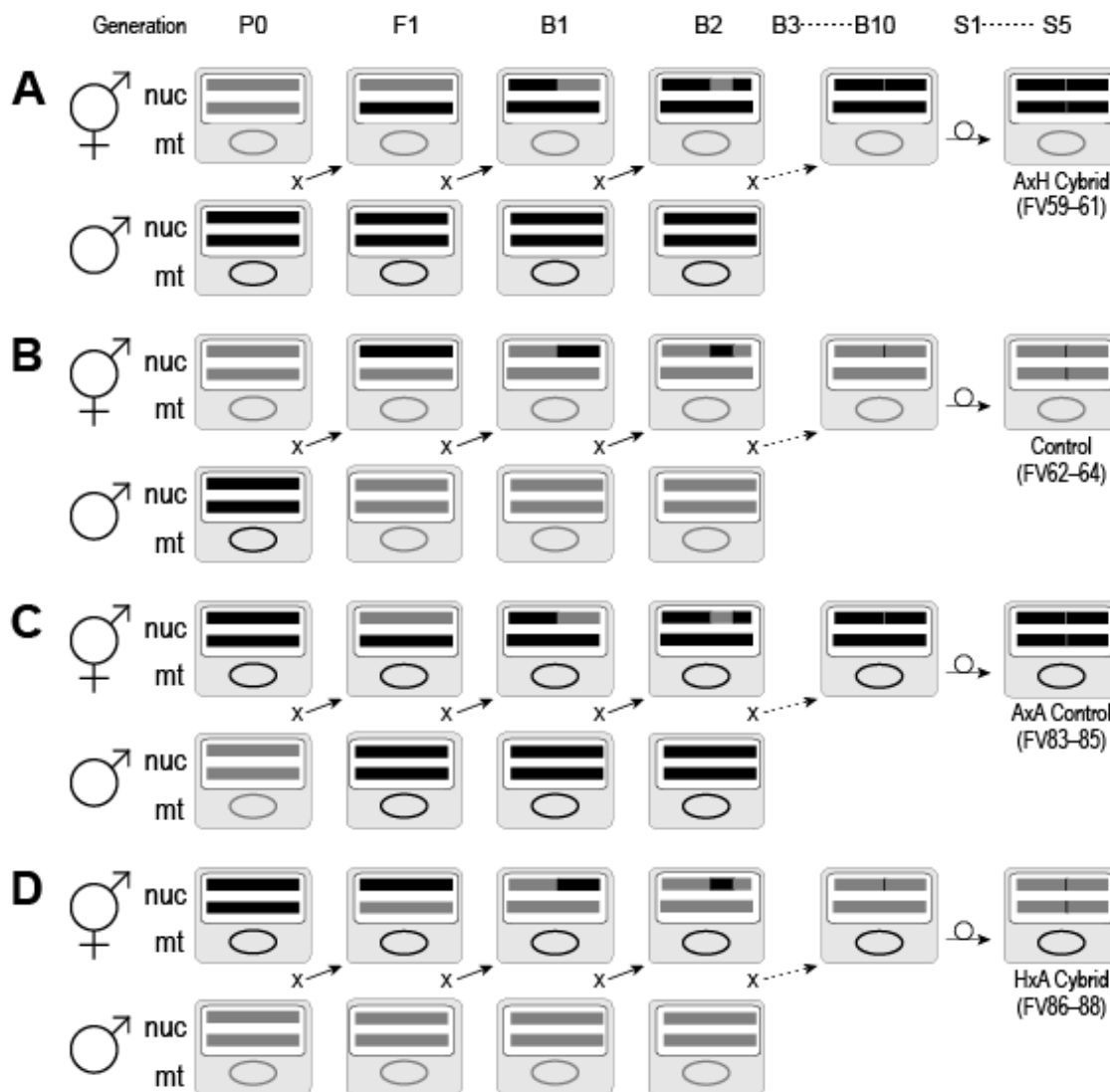
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215 Figure 1

216 Cross design and expected genotypes of lines. The average neutral expectation allele retention

217 patterns in hybrid lines are depicted using gray shading for HK104 alleles and black for AF16

218 alleles. An example diploid autosome pair ("nuc," horizontal lines) and mitochondrial genome

219 ("mt," circular genome) are depicted for the following generations: P0, F1, first and second

220 backcross generations (B1, B2), B10, and the fifth generation of serial selfing (S5). Control

221 crosses in panel B (FV62–64) and in panel C (FV84–86), in which an F1 hybrid is serially

222 backcrossed to males from the P0 maternal strain, reconstitute the P0 maternal nuclear genotype

223 on the same cytoplasmic background. Experimental crosses in panel A (FV59–61) and in panel

224   D (FV86–88), in which an F1 hybrid is serially backcrossed to the P0 paternal strain, produce

225   cytoplasmic-nuclear hybrid (cybrid) lines that reconstitute the P0 paternal nuclear genotype on

226   the maternal cytoplasmic background.

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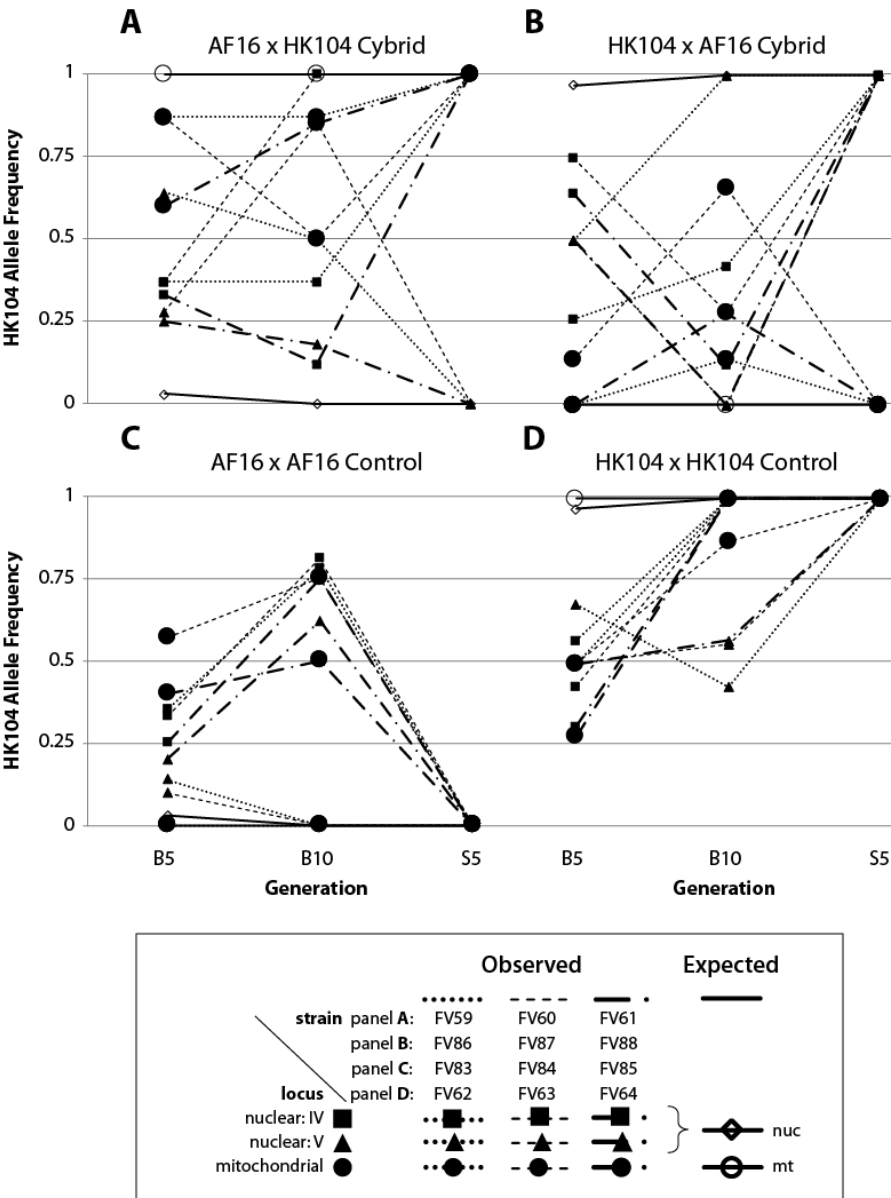
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230   Figure 2

231   HK104 allele fraction values in cybrid and control lines. The HK104 allele frequencies at two

232   nuclear loci (cb-m67(IV) and cb-m103(V)) and one mtDNA locus are shown for generations B5,

233   B10 and S5. Panels A and B contain genotype data for two reciprocal crosses that produced three

234   replicate cybrid lines each; panels C and D show data from triplicate reciprocal control lines.

235   Panel letters correspond to the cross design panel lettering in Figure 1. Each panel contains

236   eleven data series. Nine comprise three loci (distinguished by marker shapes (squares (IV),

237   triangles (V), and circles (mtDNA))) in each of three replicate lines (distinguished by dotted,

238   dashed, and dotted-dashed lines). The two expected allele frequency patterns for nuclear

239   (diamond) and mitochondrial (circle) loci are plotted as solid lines.

240

241

242

		Primers									
		cb-	m26	m205	m172	m67	m103	m124	m127	18178	34440
Line		Chr	II	III	IV	IV	V	X	X	mt	mt
Type	Name										
A AxH Cybrid	FV59		■	■	*	*	■	■	■	▦	▦
AxH Cybrid	FV60		■	■	*	*	■	■	■	▦	▦
AxH Cybrid	FV61		■	■	*	*	■	■	■	▦	▦
B AxH Control	FV62		▦	▦	▦	▦	▦	▦	▦	▦	▦
AxH Control	FV63		▦	▦	▦	▦	▦	▦	▦	▦	▦
AxH Control	FV64		▦	▦	▦	▦	▦	▦	▦	▦	▦
C HxA Control	FV83		■	■	■	■	■	■	■	■	■
HxA Control	FV84		■	■	■	■	■	■	■	■	■
HxA Control	FV85		■	■	■	■	■	■	■	■	■
D HxA Cybrid	FV86		▦	▦	*	▦	▦	▦	▦	■	■
HxA Cybrid	FV87		▦	▦	*	▦	▦	▦	▦	■	■
HxA Cybrid	FV88		▦	▦	*	▦	▦	▦	▦	■	■
P0	AF16		■	■	■	■	■	■	■	■	■
P0	HK104		▦	▦	▦	▦	▦	▦	▦	▦	▦

■ AF16 homozygosity   ▦ HK104 homozygosity

243 _____

244

245   Figure 3

246   S5 generation line genotypes. Following cybrid and control line hybridization, each line (FV##)

247   was propagated by selfing for five generations to generate homozygosity across the nuclear

248   genome (Figure 1). DNA extracted from a population of individuals from each line was

249   genotyped at nuclear and mitochondrial AFLP and RFLP markers ("cb-") on multiple

250   chromosomes ("Chr"). Control genotypes were also obtained from the P0 parental strains AF16

251   and HK104. Panel letters correspond to the cross design lettering in Figure 1. Panels A and D

252   contain the genotypes of the replicate cybrid lines; panels B and C contain the control line

253   genotypes. Genotype data are depicted by shading (black for AF16 homozygosity; gray for

254   HK104 homozygosity). White asterisks indicate genotypes that do not agree with the expectation

255   from the cross design.

256

257

258

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