Reproductive isolation in *Caenorhabditis briggsae*

Dysgenic interactions between maternal- and zygotic-effect loci result in a delayed development phenotype

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**Abbreviations:** BDM, Bateson-Dhobzhansky-Muller; VRI, variable reproductive isolation

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In sexual species, speciation occurs through the accumulation of genetic barriers to gene flow. In *Caenorhabditis briggsae*, one such barrier impedes gene flow between temperate strains and the tropical AF16 strain. Up to 20% of F2 progeny derived from crosses of AF16 to strains from the temperate clade exhibit a delayed development phenotype. This phenotype, which results from dysgenic interactions between maternal- and zygotic-effect loci, causes a ~21% decrease in the intrinsic growth rate. The maternal-effect requires contributions from both parental genotypes. The dysgenic maternal-effect allele appears to be fixed in the temperate clade of *C. briggsae* and appears to have arisen between 700 and 15,000 years ago. The dysgenic zygotic allele appears to be present only in AF16 and also may be of recent origin.

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**Introduction**

Reproductive isolation refers collectively to all genetic barriers that prevent or limit gene flow between populations. In sexual species, the advent of reproductive isolation is considered to be coincident with speciation. Early theoretical studies implicated dysgenic interactions among two or more genes within an adaptive gene complex as the genetic basis to gene flow restriction. While several models have been advanced that allow for single-gene speciation, the multigene Bateson-Dhobzhansky-Muller (BDM) model still is considered the predominant model of speciation.

Experimental support for the BDM Model has come from crosses between partially isolated species. These studies, mostly in Drosophila, have shown that multiple loci contribute to hybrid sterility and lethality. However, the molecular identities of relatively few hybrid-incomparability genes have been determined. Even rarer are molecular characterizations of pairs of interacting genes. In part, this is because the very nature of reproductive isolation precludes exhaustive genetic analyses in most species pairs. Indeed, genetic studies of reproductive isolation are possible only in species pairs in which reproductive isolation is incomplete. This severely limits the type of studies that can be done in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*.

Studies of reproductive isolation between well-established species also suffer from an inability to distinguish between hybrid incompatibilities that arose coincident with and those that arose subsequent to speciation. Thus, in these studies, the genetic basis of speciation is blurred by continued divergence. This problem can be addressed through genetic studies of incipient speciation. In *Drosophila pseudoobscura* subspecies, as few as five loci may account for sterility in F1 male hybrids. In *C. elegans*, gene flow within a 33 kb region of chromosome I is restricted by dysgenic interactions between two loci. These results indicate that speciation may occur through dysgenic interactions among small numbers of loci.

Generalization of these results from will require characterizations of incipient speciation in multiple taxa. One promising species for such studies is *Caenorhabditis briggsae*. *C. briggsae* is a cosmopolitan species that, like *C. elegans*, is a protandrous hermaphrodite. Unlike *C. elegans*, *C. briggsae* is geographically structured with at least five distinct populations. Moreover, some F2 progeny derived from crosses between the AF16 and HK104 strains of *C. briggsae* are subject to hybrid breakdown. These F2 hybrids exhibit a delayed-development phenotype that is associated with homozygosity of AF16 alleles on chromosome III. The delayed development phenotype is sufficient to distort marker transmission ratio in recombinant inbred lines.

This paper provides a more detailed characterization of the delayed-development phenotype, provides evidence for the involvement
of dysgenic heteroallelic interactions involving one or more maternal-effect genes and demonstrates that the genetic architecture responsible for this phenotype is of recent origin.

Results

Approximately 20% of F2 self-progeny obtained from F1 hermaphrodites derived from crosses between C. briggsae strains AF16 and HK104 exhibit a delayed development phenotype (Table 1). At 48 h, greater than 95% of animals scored as delayed were L2 larvae and less than 5% were L3 larvae. In contrast, their non-delayed siblings all had reached the L4 larval stage. In control crosses, nearly all AF16 and HK104 animals were L4s by 48 h (Table 1). Cross direction had little or no impact as similar results were obtained regardless of the P0 maternal strain (Table 1). Qualitatively similar results were obtained for F2 progeny derived from crosses of F1 males to F1 hermaphrodites (Table 1). However, only 13.7% of F2 cross progeny derived from P0 crosses of HK104 males to AF16 hermaphrodites were delayed. This cross was not repeated so it is not clear if this result was anomalously low or if it represents a consistent difference from the results obtained from the reciprocal cross and for F2 self-progeny.

Delayed F2s took approximately 15 h longer to reach reproductive maturity than their non-delayed siblings and the AF16 and HK104 parental controls (Fig. 1). This delay in the onset of reproduction resulted in significant decreases in intrinsic growth rates in delayed F2s (Table 2). The delayed-development phenotype is associated with homozygosity of AF16 alleles on chromosome III and one consequence of the decreased intrinsic growth rate in these animals was the under-representation of chromosome III-linked AF16 alleles in three independent sets of recombinant inbred lines derived from crosses of AF16 to HK104.\(^{22,23}\) This distortion of allele segregation ratios on chromosome III demonstrates that selection against delayed F2s is sufficiently deleterious to have an impact on population structure.

Homozygosity of AF16 alleles on chromosome III is not sufficient to explain the delayed-development phenotype. This is evident from the reproductive schedule of the AF16 parental strain, which is not delayed (Fig. 1). Rather, AF16 alleles on chromosome III must be dysgenic in combination with HK104 alleles at one or more additional loci. Several attempts to identify zygotic loci with dysgenic HK104 alleles were unsuccessful. First, several loci throughout the genome were genotyped in pools of delayed F2 hybrids. None of these loci were skewed toward HK104 homozygosity (data not shown). Second, in recombinant inbred lines derived from crosses of AF16 to HK104, no chromosomal regions were observed in which HK104 alleles were under-represented.\(^{22,23}\) Attempts also were made to construct fixed strains in which all progeny were delayed. These attempts failed as several generations of selection for delayed animals still resulted in the segregation of delayed and nondelayed progeny from individual hermaphrodites (see below).

The delayed-development involved a maternal-effect gene. This was determined from crosses of F1 hybrids to the AF16 parental strain. Delayed progeny were infrequent when F1 males were mated to AF16 hermaphrodites but frequent when F1 hermaphrodites were mated to AF16 males (Table 3), i.e., delayed progeny were obtained from heterozygous mothers that possessed HK104 alleles but not from homozygous AF16 mothers. These results also demonstrated that zygotic homozygosity of HK104 alleles was not required for the occurrence of the delayed development phenotype.

The delayed development phenotype may involve dysgenic heteroallelic interactions at the maternal-effect gene. This conclusion is based on attempts to establish a fixed strain of delayed animals. In these attempts, multiple lines were initiated, each from a single delayed F2 hermaphrodite. Delayed F2 hermaphrodites all should have been homozygous for the AF16 allele at the dysgenic zygotic locus on chromosome III.\(^{22}\) These lines then were propagated, for several (>10) generations through a single delayed hermaphrodite per generation in a attempt to fix HK104 alleles at the dysgenic maternal-effect locus. For each line, ten replicate sub-lines were established each generation. Each sub-line was scored for the presence of delayed progeny in the next generation. Delayed progeny from one sub-line then were picked to initiate the next generation. If homozygosity of HK104 alleles at a single maternal-effect locus was sufficient to cause a delayed development phenotype, then one quarter of the sub-lines in the F3 generation should have been fixed for these alleles. Delayed progeny should have been present in all F4 sub-lines from such HK104 homozygotes. However, after ten generations of selection, no strains were established in which delayed progeny were always present. One explanation for this result is that heterozygosity was required in maternal-effect genotype in order to generate delayed progeny in the next generation. In this case,
heterozygous mothers would continually segregate homozygous progeny, from which no delayed progeny would be obtained. Maternal heterozygosity may have been required because of a dysgenic heteroallelic interaction or because HK104 and AF16 alleles were required at two closely linked loci.

The allelic variants responsible for the delayed development phenotype appear to be of recent origin. This was determined from pairwise crosses between AF16, HK104 and several additional strains of *C. briggsae* and from inferences based on the phylogenetic relationships of these strains (Fig. 2). AF16 and HK104 are members of the tropical and temperate clades of *C. briggsae*, respectively. AF16 and HK104 were crossed to three additional strains from the tropical clade and eight additional strains from the temperate clade. Delayed F2s were frequent whenever AF16 was crossed to any of the temperate strains, but not when AF16 was crossed to any of the tropical strains (Table 1).

The apparent fixation of the dysgenic maternal-effect allele in the temperate clade indicates this allele arose after the divergence of the temperate clade from the tropical clade but before divergence within the temperate clade. A coalescent times of ~8.92 × 10^5 generations has been estimated for the divergence of the tropical clade from the temperate clade. A coalescent time of

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**Figure 1.** Reproductive schedules of F2 hybrids. Average daily counts at 20°C of progeny for (A) AF16 (n = 17) and (B) HK104 (n = 15) parental controls and for F2 hybrids derived from crosses of (C) AF16 males mated to HK104 hermaphrodites and (D) HK104 males mated to AF16 hermaphrodites. In C and D, the dotted lines represent the reproductive schedules of F2 hybrids that were scored as delayed at 48 h (C, n = 12; D, n = 11) and the solid lines represent the reproductive schedules of their nondelayed siblings (C, n = 27; D, n = 22).

**Table 2.** Intrinsic growth rates of *C. briggsae* parental controls and F2 hybrids

| Cross          | Hermaphrodite | Male    | \( r_{max} \)             |
|----------------|---------------|---------|---------------------------|
| AF16 x AF16    | 1.53 ± 0.03   | (17)    |
| HK104 x HK104  | 1.38 ± 0.09   | (15)    |
| HK104 x AF16   | 1.44 ± 0.06   | (33)    |
| delayed        | 1.24 ± 0.07   | (11)    |
| nondelayed     | 1.53 ± 0.06   | (22)    |
| AF16 x HK104   | 1.37 ± 0.07   | (39)    |
| delayed        | 1.17 ± 0.07   | (12)    |
| nondelayed     | 1.51 ± 0.03   | (27)    |

*Intrinsic growth rates for individual hermaphrodites were determined from reproductive schedules as described in Vassilieva and Lynch. Values reported are the means ± 2 sem. Sample sizes indicated in parentheses.*
4.5 $\times$ 10$^3$ generations has been estimated for the divergence of strains within the temperate clade. 20,21 The dysgenic maternal-effect allele(s) likely arose some time between these upper and lower boundaries.

High frequencies of delayed F2s only were observed when HK104 was crossed to AF16 and not when HK104 was crossed to other tropical strains (Table 1). Hence, the dysgenic zygotic allele on chromosome III is not fixed in the tropical clade and likely arose after AF16 diverged from VT847, PS1185 and PS1186. Of these strains, AF16 is most closely related to PS1185. The divergence of AF16 from PS1185 is 0.0005 (Fig. 2). Using a mutation rate ($\mu$) of 5.4x10$^{-9}$ mutations/generation, a divergence time, $t = K/2 \mu$, of ~4.6 $\times$ 10$^4$ generations can be estimated for these strains. 21,24-26

### Discussion

In sexual species, speciation can occur through the accumulation of genetic barriers to gene flow. 1,3,16 In this paper, one such barrier, which affects the time it takes C. briggsae F2 hybrids to reach sexual maturity, is described. At present, this dysgenic phenotype only is described as delayed development at a 48 h time point. A time course of development in delayed animals is needed for a full understanding of this developmental defect. The outcome of such studies may provide insight into the underlying genetic pathways affected in delayed F2 progeny.

The reproductive barrier that results from the delayed development phenotype is not complete. Gene flow appears to be affected only on chromosome III and even on this chromosome gene flow is limited but not precluded. 22,23 However, as speciation is thought to occur through the accumulation of multiple reproductive barriers, any dysgenic interaction that limits gene flow has the potential to contribute to a speciation event. 3,18,27

Dysgenic interactions between at least two loci are involved in the delayed development phenotype. The first of these is a locus with a maternal effect. The involvement of maternal-effect genes in reproductive isolation is not unusual. Snail species often are isolated by chirality, which, in several taxa, is determined by maternal-effect genes. 7,20 Drosophila simulans alleles of the maternal-effect mhr gene are dysgenic in combination with D. melanogaster alleles of the zygotic zhr genes. 29,30 This interaction results in embryonic lethality in female hybrids. Maternal-effect genes also have been implicated in the control of allochronic mating differences between the corn and rice strains of the fall armyworm, Spodoptera frugiperda. 31 What is unusual is that the maternal effect involved in the delayed development phenotype appears to require maternal contributions from both parental genotypes. These AF16 and HK104 maternal-effect loci appear to be on the same chromosome and possibly are allelic. The HK104 allele of this locus appears to be fixed within the temperate clade of C. briggsae as delayed F2 progeny were observed in all crosses of AF16 to any of the temperate strains tested. A coalescent time of ~4,000 generations has been estimated for the temperate clade of C. briggsae, which means that this allele may have arisen as recently as 700 y ago. 20

The second locus involved in the delayed development phenotype had a zygotic effect and was located on chromosome III. 22 The dysgenic allele of this locus did not appear to be fixed within the tropical clade as delayed F2s were frequent only when HK104 was crossed to AF16. (Alternatively, the dysgenic allele at the zygotic locus may be fixed in the tropical clade if a dysgenic tropical allele was not fixed at the maternal-effect locus.) The dysgenic allele on chromosome III also appeared to be absent from the ED3101 strain, which is a member of the Nairobi clade of C. briggsae. 21,32 The presence of the dysgenic zygotic-effect allele only in AF16 indicates that this allele also is of recent origin. As the tropical clade of C. briggsae exhibits considerable structure, it should be possible to refine estimates for the origin of this
dysgenic allele by crossing HK104 to additional tropical strains and scoring these crosses for delayed F2 progeny.21

The conclusion that the dysgenic alleles involved in the delayed development phenotype are of recent origin is based on the tropical and temperate clade divergence averaged across several loci located throughout the the *C. briggsae* genome (Fig. 2).20,21,33 However, localized regions of the genome and hence these dysgenic alleles may differ considerably from the genome average in divergence. For example, divergence within the *peel-1/zeel-1* haplotype domain is 50-fold higher than genome-wide average divergence in *C. elegans*.14 Therefore, a rigorous dating of the dysgenic *C. briggsae* alleles involved in the delayed development phenotype must wait until the molecular identities of these genes are known.

The skewed chromosome III segregation ratios obtained in three sets of recombinant inbred lines demonstrate that the delayed development phenotype is maladaptive.22,23 However, the tropical and temperate populations of *C. briggsae* are geographically isolated and while migration rates between these populations are not known, they have not been sufficient to “disrupt the high differentiation and linkage disequilibrium among multilocus haplotypes.”20,21,32 In the absence of gene flow between these populations, the dysgenic alleles that result in the delayed development phenotype likely have not been exposed to negative selection. The origin of this dysgenic interaction between the allopatric tropical and temperate populations is consistent with the BDM model of speciation.1,3,5,6

The delayed development phenotype also is consistent with the genic and variable reproductive isolation (VRI) models of speciation.27,33 The genic model describes four stages of speciation: (1) population differentiation without reproductive isolation; (2) further population differentiation with some mal-adaptive hybrid genotypes; (3) gene flow restricted between populations by reproductive isolation throughout much but not all of the genome and; (4) reproductive isolation complete with no gene flow between populations.27 The delayed development phenotype clearly is maladaptive but restricts gene flow only on chromosome III. As such, it falls into stage ii in the genic model. VRI posits that posits that dysgenic BDM alleles will be polymorphic in partially isolated populations for significant periods of time.33 This clearly is the case for the delayed development phenotype as the dysgenic allele of the zygotic gene was apparent only in AF16 and not in the other tropical strains that were tested. One issue relevant to VRI is how much do polymorphic reproductive incompatibilities contribute to reproductive isolation over the course of speciation. To answer this question for the delayed development phenotype, it will be necessary to (1) determine the prevalence of the dysgenic zygotic allele within the tropical clade and (2) to determine whether or not gene flow of haplotypes linked to this dysgenic allele is suppressed in the temperate clade.

It also will be of interest to determine whether or not the dysgenic alleles involved in the delayed development phenotype reside in regions of the *C. briggsae* genome that recently have been subjected to positive selection. Dysgenic alleles involved in BDM incompatibilities can become fixed through neutral mechanisms.1,2,5,6 However, they also may become fixed due to geographically restricted positive selection.36,33 Recent selective sweeps throughout much of the genome of *C. elegans* have been documented.34 The occurrence of selective sweeps within *C. briggsae* populations remains to be tested.

A dysgenic interaction that partially restrict gene flow also has been identified in *C. elegans*.14,35 This interaction, which is between two closely linked genes, *peel-1* and *zeel-1*, results in embryonic lethality.15 As with the delayed development phenotype in *C. briggsae*, the *peel-1/zeel-1* interaction is between a zygotic-effect gene and a parental-effect gene. However, *peel-1* has a paternal- rather than a maternal-effect.14 Also unlike the genes involved in the *C. briggsae* delayed development phenotype, *peel-1/zeel-1* haplotypes are not restricted to distinct geographic populations. Indeed, the maintenance of *peel-1/zeel-1* haplotypes appears to result from balancing selection and chromosomal regions that flank the *peel-1/zeel-1* haplotype domain recombine freely.14 Once the loci involved in delayed development have been identified, it will be of interest to compare the evolutionary histories of these dysgenic interaction in these two species of *Caenorhabditis*.

**Materials and Methods**

**Strains and strain maintenance.** AF16 and VT847 were obtained from the *Caenorhabditis Genetics Center*, HK104 and HK105 from H. Kagawa, PS1185 and PS1186 from P. Sternberg, JU279, JU383, JU439 and JU441 from M.-A. Felix and PB800, PB826 and PB859 from local collections; PB800 from decaying mushrooms in Dayton OH, PB826 from a snail obtained from Hueston Woods State Park, College Corner OH and PB859 from a snail obtained from the Wright State University campus woods, Dayton OH. All strains were grown at 20°C on lawns of *E. coli* strain OP50.

**Experimental crosses and intrinsic growth rate determinations.** Crosses were conducted between males and sperm-depleted hermaphrodites.35 F1 hermaphrodites were allowed to lay eggs for one hour and then removed. 48 h later, F2 progeny were counted and scored for the delayed development phenotype. Individual F2 progeny were transferred to new plates daily (beginning at 48 h) through day 6. F3 progeny were counted and removed from these plates before they reached adulthood. Intrinsic growth rates were determined from daily brood counts as described by Vassilieva and Lynch.36

**DNA sequence analyses.** Amplification primers (Table 4) were designed based on the AF16 genome sequence.37 Amplified products were commercially sequenced. Sequence results were trimmed to include data only from introns. A maximum likelihood phylogeny of the *C. briggsae* strains used in this study was constructed using the “One Click” site at Phylogeny.fr (www.phylogeny.fr/version2.cgi/index.cgi) to analyze a concatenated data set of 4,002 nucleotides.38,39 The divergence (K) of AF16 from PS1185 was determined from the length of the branch that separated these two strains. The divergence time (T) of AF16 from PS1185 was estimated as t = K/2μ, using a mutation rate (μ) of 5.4 × 10⁻⁹ mutations/generation for *C. briggsae*.34,36
Table 4. Amplification primers

| Gene     | Forward      | Reverse          |
|----------|--------------|------------------|
| Cbr-mab-20 | TGC TCT TGT GGA ATG CGA C | CGC TTT TTT GTG TTG ATG GTG GG |
| Cbr-tra-2  | GCA ACT ACA CCG TCA GAA TGC AC | TAT GCC GAG CCC ACT CTT TG |
| Cbr-tra-3  | CAT CTT TTT GTG GAA GGA GCA TCG C | TGA TAC ACC TCT CTT TGC GCC CG |
| Cbr-mes-2  | CAG GCC AAA AAT GCT GTG ACA AGT G | AAT CGG TGC TGT TGA TGG AGG C |
| Cbr-glp-1  | CAT GGC AAA GGA GTG CGA GAA GGC | CGA AAC CAC ATC CAA CGA AGC |
| Cbr-her-1  | CGT CAG TCA TGG ATT GGT CGG | CAT CTA CTC GGA GAG ACA GTT CGG |
| Cbr-daf-3  | TGA ATG TAG CTT CTT TGT TGG TGG | TAC CCT TCT TCG GAA CTC GTG C |
| Cbr-ram-5  | TGG GGA AAG TAG CAC AAC ACC | TCG TCA AAT CTC CAG TCT GCG |

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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