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Research paper

Hybridization is limited between two lineages of freeze-resistant *Trichinella* during coinfection in a mouse model

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ABSTRACT

Hybridization between two closely related but distinct genetic lineages may lead to homogenization of the two lineages with potentially novel phenotypes, or selective pressure to avoid hybridization if the two lineages are truly distinct. *Trichinella nativa* and *Trichinella* T6 are zoonotic nematode parasites which can be distinguished genetically despite occasional hybridization. Here, using an experimental murine model, we attempt to determine whether there are barriers to hybridization when sizeable numbers of each lineage are allowed to coinfect a host. Two mice were independently infected with equal numbers of *T. nativa* and T6. The offspring of these coinfections were genotyped at two microsatellite loci and one mitochondrial locus capable of distinguishing *T. nativa* from T6 genotypes. Among larvae in the F1 generation, offspring of every possible mating were encountered. Most larvae (63.6%) derived from *T. nativa* × *T. nativa* matings, while 21.1% of offspring were the product of T6 × T6 matings, and only 15.3% were hybrid offspring of *T. nativa* × T6 crosses, differing markedly from null expectations. In this experimental model, *T. nativa* and *Trichinella* T6 were able to mate, but ratios of offspring indicated pre- or post-zygotic barriers to hybridization that may include assortative mating, genetic incompatibilities, and/or differences in the fitness of offspring. These barriers would limit gene flow between these two lineages in a natural setting, serving as a barrier to their homogenization and promoting their persistence as distinct and separate entities.

1. Introduction

Closely related but genetically distinct biological lineages are sometimes able to interbreed (see Rieseberg and Carney, 1998, Scribner et al., 2001, and Detwiler and Criscione, 2010 for reviews of specific phyla). This may result in gene flow, leading to the homogenization of the two lineages or extirpation of one lineage in favor of the more successful phenotype (Rhymer and Simberloff, 1996). Alternatively, if lineage phenotypes are adaptive, behavioral or physiological barriers may maintain distinctions between lineages (Haldane, 1948). Absolute reproductive isolation would meet the criteria for one influential species concept (Mayr, 1942). Thus, the results of mating between two such lineages may be interpreted along a spectrum from intra-specific population structure to inter-specific reproductive isolation, including the possibility of incipient speciation. Distinguishing among these outcomes requires an understanding of both the biology and demography of the populations in question. Examining the outcomes of experimental crosses between genetically distinct representatives may provide insight into the biological relevance of hybridization and introgression of genes.

Trichinella nativa and the *Trichinella* T6 (henceforth referred to as T6) genotypes are genetically distinct lineages of nematode parasites endemic to the Canadian Arctic, peculiar for their freeze resistance. The two cannot be distinguished from each other by any morphological character (Pozio and Zarlenga, 2005). T6 is currently considered a sister taxon to *T. nativa*, but has not been raised to species status due to its similarities with *T. nativa* (Pozio et al., 1992; Zarlenga et al., 1999, 2001; La Rosa et al., 2003; Reichard et al., 2008). Both lineages are primarily found in wild carnivores, such as foxes and wolves (Kapel, 2000), and complete their life cycle within a single host, with developmental stages being separated only by tissue type (Reichard et al., 2008). Despite their similarities, *T. nativa* and T6 may be differentiated using genetic markers. This implies that there has been a history of separation between the two (Pozio, 2000), most likely during the Pleistocene glaciations (Zarlenga et al., 2006). Previously, T6 was thought to occupy a distinct geographic range from that of *T. nativa*, but the lineages have been found together in particular host populations (Reichard et al., 2008; La Rosa et al., 2003). Furthermore, natural

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T. nativa/T6 hybrids have been observed (La Rosa et al., 2003, Dunams-Morel et al., 2012), raising the possibility of limited gene flow. The extent and biological consequences of introgression between the two lineages remain unknown.

Hybridization and subsequent introgression erodes species boundaries. Dunams-Morel et al. (2012) distinguished natural *T. nativa*/T6 hybrids from true-breeding individuals in each lineage using genetic markers, suggesting that in the studied population, introgression had not been extensive. This may be for lack of opportunity, if the taxa only rarely co-occur (Reichard et al., 2008). Changes in the geographic distribution of lineages, as are expected in changing climates, may provide more opportunities for breeding between closely related lineages, and an increase in gene flow. Increased gene flow between two such lineages might produce a single lineage with greater genetic and/or phenotypic diversity.

By contrast, introgression may be limited, in spite of opportunities for hybridization, by pre- or post-zygotic barriers. La Rosa et al. (2003) showed that when pairs of *T. nativa* and T6 were compelled to hybridize, fewer offspring resulted than in purebred lines of either lineage. Moreover, hybrid F1 individuals descended from *T. nativa* mothers did not produce offspring when mated to each other. Such reproductive penalties should reinforce reproductive isolation between *T. nativa* and T6. Pre-zygotic barriers for parasites include geographic isolation, isolation by host-specificity, and mate recognition systems (Southgate et al., 1998). As *T. nativa* and T6 currently have overlapping geographical ranges and host species, mate recognition provides the most likely pre-mating barrier to hybridization between these two lineages. This process, termed assortative mating, occurs where particular parasites mate preferentially with their own lineage despite access to another competent for hybridization (Wright, 1921). Such behavior reinforces inbreeding, promoting genetic divergence among populations. Differentiation of lineages that occurs due to any form of pre-zygotic isolation may give rise to genetic incompatibilities that reduce the fitness of hybrid offspring, resulting in post-zygotic barriers to hybridization. Adaptations that minimize such maladaptive hybridization would increase a pure-breeding individual's fitness as measured by the average number of viable offspring produced from a mating event (relative fitness). This process of reinforcement may be important in cementing evolved differences between incipient species (Kelly and Noor, 1996; Noor, 1999; Matute, 2010).

Here, using an experimental murine model, we attempt to determine the likely outcome of hybridization between these two lineages when encountered in a well-mixed population. We characterized the nature and frequency of hybridization in experimental coinfections involving many individuals from both the *T. nativa* and T6 lineages, where each parent was capable of "choosing" similar or dissimilar mates. We genotyped offspring of this initial cross to determine the relative contributions of each parental lineage, and to assess the degree of admixture occurring under these experimental conditions. In order to track the inheritance of somatic and maternally inherited genes, we employed two nuclear microsatellite loci and a mitochondrial restriction fragment length polymorphism.

2. Methods

2.1. Sample preparation

In May 2012, two Swiss-Webster mice (NCI) were experimentally infected, each with approximately 420 larvae consisting of equal numbers of *T. nativa* (ISS45) and the T6 genotype (ISS34). Outbred mice were used in order to replicate experiments conducted previously (La Rosa et al., 2003). The introduced larvae migrated to the gut, wherein they matured and mated. The F1 generation then migrated to the animals' skeletal muscle tissue. After 49 days, muscle larvae from one mouse (henceforth referred to as mouse A) were obtained by digesting the skinned and eviscerated carcass in a 1% HCL/1.0% pepsin solution. Immediately after isolation, 500 larvae from this mouse digestion were used to infect 5 uninfected mice in order to follow the success and mating tendencies of the F1 generation. Data from subsequent generations (F2 and F5) are presented as supplemental information as there was no replication of these data. The remaining worms from mouse A (those not used for infection) were saved for genetic analysis. The second mouse (mouse B) was sacrificed 70 days post-infection, and muscle larvae were isolated as before and saved for genetic analysis, but no new mice were infected.

From the F1 generation, individual larvae were isolated from their respective pooled worm suspension (mouse A or B) by micropipette utilizing a dissection microscope for visualization. Individual worms were stored at 4 °C in water until ready for DNA extraction. DNA was purified from these single larvae using the DNA IQ System Tissue & Hair Extraction Kit (Promega Corp.) according to manufacturer recommendations. Individual DNAs from mouse replicates in the F1 generation were kept separate in order to evaluate repeatability of the experimental crosses.

In order to control for fitness differences between the parental strains, reproductive capacity indices (RCI) were calculated from mice infected with either strain alone. In each case, five mice were infected with 500 muscle larvae of *T. nativa* or T6. After six months, muscle larvae were collected from infected mice using the method described above, and pooled in 25 ml water for counting. Three separate counts of a 200 μl sample were conducted to enumerate the muscle larvae collected, and the average was reported. The resulting pools (*T. nativa* or T6) were used to infect an additional five mice, and counts were repeated after seven months of infection. RCI was calculated as the average number of worms recovered from two replicates of each genotype divided by the number of worms used to infect host animals.

2.2. Amplification of loci

2.2.1. Microsatellites

Amplification of two nuclear loci and one mitochondrial locus was attempted for 93 larvae from each pool (93 from mouse A, 93 from mouse B, 93 from the F2 generation, and 93 from the F5 generation). Individual larval DNAs were amplified for 2 microsatellite loci: TP32 and TP47 (Rosenthal et al., 2008). Larval *T. nativa* and T6 parental strains had been previously genotyped and determined to differ at these loci (see Dunams-Morel et al., 2012). Therefore, hybrids could be identified by the coincidence of alleles particular to each parental type. Microsatellite loci were amplified by 20 μl PCR reactions conducted in 96 well plates. Each reaction contained 5 μl of template DNA, 0.5 mM dNTPs, 0.5 μM of each primer, 0.5 U Platinum High Fidelity Taq polymerase (Invitrogen), and 2.5 mM $MgSO_4$ in 1× High Fidelity PCR buffer (Invitrogen). Reactions were subjected to thermal cycling as follows: 94 °C for 3 min, then 40 cycles of 94 °C for 30 s, 53 °C for 45 s and 68 °C for 1 min, with a polishing step of 68 °C for 5 min. In every reaction plate, no template, parental *T. nativa*, and parental T6 controls were included. For size fragment analysis on an ABI 3130 capillary electrophoresis machine, PCR reactions were diluted 1:6 and 1 μl of diluted microsatellite PCR product was mixed with 9 μl HiDi Formamide containing 0.2% GeneScan 500 LIZ molecular weight standards (Applied Biosystems). The resulting size fragment trace files were viewed in Geneious (Biomatters Ltd.), and alleles were called based on fluorescence peaks allowing the software to call each peak in order to avoid human bias.

2.2.2. Mitochondrial DNA

The matrilineage of each larva was determined using a mitochondrial specific restriction fragment length polymorphism (RFLP). Mitochondrial DNA (mtDNA) from each larval DNA extract was amplified using Trich-COB F1 and Trich-seq R3 primers (Lavrov and Brown, 2001) resulting in PCR products of 960 base pairs (bp). Each 20 μl PCR reaction contained 5 μl template, 2.5 mM $MgSO_4$, 0.5 mM dNTPs, 50 nmol of each primer, and 1 unit HiFi Platinum Taq (Invitrogen). Amplified DNA was