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Rare but evolutionarily consequential outcrossing in a highly inbred zoonotic parasite

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ABSTRACT

Recurrent self-mating can result in nearly clonal propagation of biological lineages, but even occasional outcrossing can serve to redistribute variation in future generations, providing cohesion among regional populations. The zoonotic parasite *Trichinella spiralis* has been suspected to undergo frequent inbreeding, resulting in genetically uniform larval cohorts which differ markedly from one another. Here, we explored the extent of inbreeding for this parasite by determining how genetic variation (at variable microsatellite markers) is distributed among 1379 larvae derived from 41 wild boars in Extremadura, Spain. In particular, we sought to determine how much of the genetic variation in this region's parasites occurs among the larvae of any given wild boar, and whether each derives from one, or more, parental lineages. We found strong evidence for inbreeding, resulting in genetically distinct parasite subpopulations among the parasites derived from many pairs of wild boar. Fully two-thirds of these parasite cohorts appear to derive from inbred parents; in 10% of the wild boars, parasites were so inbred as to become absolutely fixed in all of the assayed genetic loci. In spite of this, more than one pair of parents appear to have given rise to the infections in one-third of the sampled wild boars, resulting in mixed infections. These mixed infections should slow losses of heterozygosity and multi-locus polymorphism in any given parasite lineage. Such outcrossing should limit distinctions that would otherwise accumulate among transmission chains, thereby enforcing cohesion through the region's population in spite of its marked departure from panmixia. Conditions of transmission may differ in other regions, where such epidemiological features may engender different evolutionary outcomes.

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1. Introduction

Recurrent self-mating can result in nearly clonal propagation of biological lineages. Within such lineages, allelic variability and heterozygosity wanes; consequently, such lineages can grow ever more distinct from one another as each comes to fix distinct genetic attributes not generally shared throughout the broader population. By contrast, even occasional outcrossing can serve to redistribute genetic polymorphisms, providing cohesion among the constituents of a regional population. Facultative or obligatory self-mating has been described for certain nematodes and in a wide range of organisms including, fungi, plants, and fish

(Nordborg et al., 2002; Zauner et al., 2007; Tatarenkov et al., 2009; Bomlies et al., 2010; Beadell et al., 2011; Heitman, 2015; Kanamori et al., 2016).

The short- and long-term effects of recurrent inbreeding can be examined by estimating the reduction in regional genetic variation attributable to local cohorts. Do these cohorts encompass the range of variation present regionally, or does each represent a narrow subset, derived from inbred parents? In the case of diploid zoonotic parasites that reproduce sexually, but often as the result of self-mating, this question can be framed in epidemiological terms: how often does a given host acquire infection from a single exposure to genetically uniform parasites? Conversely, does outcrossing among these parasites (attributable to multiple, contemporaneous exposures) occur frequently enough to prevent the formation of distinct, enduring strains? Parasites in the genus *Trichinella* afford the means to address these questions, because a host's cumulative

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history of exposure can be estimated from the genetic attributes of the larvae residing in its musculature.

Parasites in the genus *Trichinella* comprise 12 known taxa occurring on all the continents excepting Antarctica (Pozio and Zarlenga, 2013). In most regions, these parasites circulate among wild carnivores and omnivores, and more rarely they infect free-ranging and backyard pigs and other domestic animals (Gottstein et al., 2009; Pozio, 2014). A scheme of the *Trichinella* cycle is shown in Supplementary Fig. S1.

Parasite transmission occurs when one or more animals feed on the carcass of an infected animal. Upon ingestion, larvae in the muscle of the consumed host develop, in the gut of the consumer, to sexually mature adults within 48 h. Once fertilised, adult females produce newborn larvae for a period of weeks (until immune responses expel adults from the gut). Newborn larvae migrate into lymphatic vessels, enter the blood vessels, and penetrate striated muscle cells; in approximately 15 days, these develop into long-lived muscle larvae which remain infective until the cycle begins anew once ingested by a subsequent host (Pozio, 2007).

Many factors may influence the prevalence and diversity of infections. Hunting and scavenging behaviours, host and prey density, the typical size of meals together with human activities (i.e. hunting customs, husbandry of susceptible domestic animals, farming practices, availability of food resources originating from the human activities) may play a role (Pozio and Murrell, 2006; Gottstein et al., 2009; Pozio, 2014). The persistence of larvae in putrefying flesh is also influenced by environmental temperature and humidity; high humidity and low temperatures favour survival even when the muscle tissue is completely liquefied (Pozio, 2016).

Marked regional differences have been discovered in the genetic variability of the principal agent of human trichinellosis, *Trichinella spiralis*. Analyses of allozymes (La Rosa et al., 1992), mitochondrial DNA and nuclear microsatellites (Rosenthal et al., 2008; La Rosa et al., 2012) have underscored exceptionally limited genetic variability among European and American populations of *T. spiralis*. Indeed, one such study found that larvae derived from 17 infected animals throughout Europe, North America, and South America encompassed no more diversity than was true of larvae derived from a single infected animal in Asia (La Rosa et al., 2012).

Further reductions in variability were recognised among the larvae derived from each host in Europe. The genetic variance among those larvae sampled from a given host was half the variance among larvae sampled at random (average pairwise $F_{st} = 0.535$, range: 0.000–0.904) (La Rosa et al., 2012). Evidently, closely related parents give rise to larval cohorts that are even more closely related than would be expected for full siblings derived from freely interbreeding parents. Such marked inbreeding might be expected if, as has been generally observed, naturally infected hosts tend to harbour low larval burdens (<1.0 larvae/g) (Zimmerman and Zinter, 1971; Malakauskas et al., 2007; Pozio and Zarlenga, 2013; Gómez Morales et al., 2014). Ingesting only a few larvae would limit any given transmission event to only a few parents. Further reductions in diversity might result if the ingested larvae are characterised by a skewed sex ratio; if immune responses reduce the likelihood of superinfection, the cumulative larval burden in a host might remain highly homogeneous. Successive rounds of such transmission would reinforce the pattern, reducing the genetic variability of parasites within each host (and augmenting the differences of parasites among such hosts). Genetic drift, engendered by strong, recurrent bottlenecks, would describe the evolutionary process in a more general sense (La Rosa et al., 2012).

Occasional outcrossing would, however, redistribute genetic variation among otherwise subdivided lineages. Evidence for this

was found when one of 22 hosts was shown to harbour larvae clearly derived from more than one exposure to *T. spiralis* (La Rosa et al., 2012). Moreover, mixed species infections in wild and domesticated hosts are often reported (Pozio et al., 1997, 2009; Malakauskas et al., 2007; Airas et al., 2010; Pannwitz et al., 2010; Bilska Zajac et al., 2013; <https://trichinella.iss.it/>) and this process has recently been shown to result in interspecific introgression (Franssen et al., 2015). Thus, even rare mixed infections may undermine distinctions that would otherwise arise among *T. spiralis* subpopulations infecting individual hosts.

Here, we sought to characterise the balance of forces contributing to, and undermining, genetic distinctions among natural infections of *T. spiralis* in an important wildlife host (wild boar) in a particular regional setting (Extremadura, Spain). Against a backdrop of low regional variation, we sought to determine how frequently hosts harbour larvae descended from more than one pair of parents. In so doing, we sought to link epidemiological processes with their evolutionary consequences, so as to better understand the implications of recurrent inbreeding in this important zoonotic parasite.

2. Materials and methods

In the present work, we conceive “subpopulation” (see Margolis et al., 1982) as meaning the larvae derived from the striated muscles of an individual host, and therefore to be synonymous with “isolate” as these larvae are often described.

2.1. Investigated area and sampling

Trichinella spiralis subpopulations (SPs) were collected from 41 wild boars (*Sus scrofa*) hunted in the Extremadura region of Spain in 2009–2010 (Table 1; Fig. 1). Muscle larvae were collected by artificial digestion using previously described procedures (European Commission, 2005). After digestion, living larvae were washed several times in PBS at room temperature and then fixed with 90% ethanol and stored at -20°C . Since three species of *Trichinella* occur in Spain and can co-occur in the same host (Zamora et al., 2015), each larva was diagnosed to species by multiplex PCR to exclude, for present purposes, individuals of either *Trichinella britovi* or *Trichinella pseudospiralis* (Zarlenga et al., 1999 as modified by Pozio and La Rosa, 2010).

2.2. Microsatellite analysis

Total DNA was purified from single larvae as previously described (La Rosa et al., 2012). Single larvae were individually genotyped by the screening of nine microsatellite loci: TS103, TS128, TS130, TS1122, TS1131, TS1380, TS1444, TS1007 and TS1010B. Primers for the first seven microsatellites have been previously described (La Rosa et al., 2012), whereas primer pairs of the TS1007 and TS1010B microsatellite loci are: (i) TS1007, 5'-ACC AAT CGA ATA GAG ATA TTT GAA T and 5'-TGA TTA AAT TGC TTT CTA CTT CTT with expected amplicons ranging from 171 to 177 bp; and (ii) TS1010B, 5'-CAT TAA CGA TGT GCT ATT TAA CGC T and 5'-TCA ATT CAT TTC ATT TCA ATC TGC G, with expected amplicons ranging from 244 to 258 bp. These two microsatellites were already identified in the course of work carried out previously for the other microsatellites (La Rosa et al., 2012), but those had not been tested. PCR conditions for all nine markers were previously described (La Rosa et al., 2012).

Genotyping was accomplished by capillary electrophoresis of PCR products using the Qiaxcel device (Qiagen GmbH, Hilden, Germany) as previously described (La Rosa et al., 2012). Allele size was determined by comparing each peak to references, the sizes of

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