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Genetic exchange and emergence of novel strains in directly transmitted trypanosomatids

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ABSTRACT

The breeding structure of protozoan infections, i.e. whether and how frequently parasites exchange genes (“sexual reproduction”), is a crucially important parameter for many important questions; it also matters for how new virulent strains might emerge. Whether protozoan parasites are clonal or sexual is therefore a hotly debated issue. For trypanosomatids, few experimental tests of breeding structure exist to date and are limited to the vector-borne human diseases *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. We infected the natural host (*Bombus terrestris*) of the monoxenous parasite *Crithidia bombi* (Trypanosomatida) either with a single strain of the parasite or in mixed infections and tested for genetic exchange among co-infecting strains using microsatellite markers. We show that strains regularly exchange genetic material, with occasional self-crossing during mixed infections. Most offspring clones fit the expected allelic pattern from a standard Mendelian segregation. In some cases, alleles are lost or gained, leading to an entirely new genotype different from either parent. Genetic exchange in *C. bombi* therefore does occur and the process also leads to allelic loss or gain that could result from slippage during recombination. The majority of novel offspring types correspond to a recombination of parental alleles. The case of *C. bombi* demonstrates that directly transmitted, monoxenic trypanosomatids can also exchange genes. Sex therefore seems to be found in very different lineages of the trypanosomatids. Furthermore, the data allowed estimating a frequency at which *C. bombi* shows genetic exchange in populations.

1. Introduction

Trypanosomatid parasites are the cause of many important diseases of humans, crops, and livestock. Examples are leishmaniasis, sleeping sickness, Chagas disease, and hartrot in palms (Dollet, 1984). An important step towards the still elusive goal of controlling such diseases is to understand the population breeding structure, i.e. whether and how frequently genetic exchange occurs. This structure is a key to understanding the epidemiology, the evolution of virulence or drug resistance, and the emergence of new pathogens (Heitman, 2006). Indeed, with the progress in analytical methods and molecular technologies the population structure of protozoan diseases has become a very active area of research whose various aspects have been reviewed several times recently (Archie et al., 2008; Criscone et al., 2005; de Meeus et al., 2006; Halkett et al., 2005; Monis et al., 2005; Smith et al., 2006), and is sometimes named ‘molecular epidemiology’ (Tibayrenc, 2005). The population structure and breeding scheme is a key to understanding the epidemiology of a pathogen, or the evolution of virulence and drug resistance (Heitman, 2006). In particular, genetic exchange among strains or sub-species is thought to be important for the emergence of novel types that can trigger an epidemic or cause severe disease; examples include *Toxoplasma gondii* (toxoplasmosis, Boyle et al., 2006; Sibley and Ajioka, 2008; Grigg and Sundar, 2009), *Plasmodium falciparum* (malaria, Gauthier and Tibayrenc, 2005), or *Trypanosoma cruzi* (Chagas disease, Macedo and Pena, 1998).

The ‘clonal theory’ of parasitic protozoa suggests that protozoans, such as trypanosomatids, are essentially clonal based on the general observation of strong linkage disequilibria and departures from Hardy–Weinberg frequencies among isolates. Rare episodes of genetic exchange among ‘clonal’ lines are accepted but it is thought to be too infrequent to break the prevailing pattern of clonal population structure (Tibayrenc and Ayala, 2002). Consequently, new variants can only arise by mutation events rather than by exchange among existing lines. The understanding of ‘strains’ and ‘clonal populations’ has slowly but steadily increased over the last decades. For example, parasite populations are now understood as being highly structured by variation among individual hosts; populations can thus be in linkage disequilibrium

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even when genetic exchange is common. Furthermore, host structure poses an 'iceberg problem', since only a subset of all strains in a population are accessible when extracting isolates from a host population (Heitman, 2006; McKenzie et al., 2008; Tibayrenc, 1999).

In many cases, the genetic population structure has been studied to identify signatures of genetic exchange. For example, persistent linkage disequlibria have been interpreted as a sign of clonality (Tibayrenc, 1999). On the other hand, lack of linkage disequlibria, allelic sequence diversity, extensive heterozygosity, contrasting phylogenies for different loci within genotypes, reassortment of blocks of sequences, or genetic hybrids among parasite variants have been suggestive of genetic exchange. Examples include *Giardia duodenalis* (= *G. lamblia*, Cooper et al., 2007; Lasek-Nesselquist et al., 2009), *P. falciparum* (Mzilahowa et al., 2007), *Theileria parva* (Katzer et al., 2006), or the trypanosomatid *Leishmania infantum* (Chargui et al., 2008). So far, direct experimental evidence for genetic exchange in protozoans is very rare. Notably in the trypanosomes and trypanosomatids, genetic exchange has only been shown in three cases so far. These studies include co-infections of the relevant vectors: different strains of *Trypanosoma brucei* in the tsetse fly, *Glossina moritans* (Jenni et al., 1986; MacLeod et al., 2005), and *Leishmania major* co-infecting the sandfly, *Phlebotomus dubosqi* (Akopyants et al., 2009). Furthermore, *T. cruzi* has been experimentally co-infected into mammalian cell lines (Gaunt et al., 2003b). In the latter case, no exchange seems to occur in the vector (triatomine bugs). However, no experimental evidence for genetic exchange was found for *Crithidia fasciculata* in mosquitoes (*Culex pipiens*, *Aedes aegypti*, Votypka et al., 2001).

Even though the empirical evidence is limited, all trypanosomatids where genetic exchange has so far been experimentally demonstrated depend on a vector (flies, bugs) for transmission and are parasites that ultimately infect mammals. In two cases (*T. brucei*, *L. major*) genetic exchange occurs in the insect vector whereas in *T. cruzi* exchange occurs in the mammalian hosts. Here, we show that genetic exchange also occurs in a monoxenic, directly transmitted extra-cellular parasite of insects, *Crithidia bombi* (Lipa and Triggiani, 1988). *C. bombi*, found in a wide range of hosts of the genus *Bombus* (bumblebees), is a flagellated kinetoplastid trypanosomatid that infects its host *per os*, resides in the hind gut, and is passed out after a few days in the faeces. It is highly virulent, since queens being infected in spring (the critical stage of the host's life cycle) lose up to 40–50% of their normal fitness (Brown et al., 2003). *Bombus* spp. are primitively social insects where the queens found colonies in spring, which subsequently grow in worker numbers until reproduction in summer. Only the mated daughter queens can hibernate and so carry the infections to the next year. Furthermore, the parasite is prevalent in natural populations with 10–70% of all individuals infected (Imhoof and Schmid-Hempel, 1999; Shykoff and Schmid-Hempel, 1991), about 40% of them by more than one strain (Salathé et al., Tognazzo et al., in preparation). We here use 'strain' to denote a given multi-locus genotype as given by several highly polymorphic micro-satellite loci. The exact genetics of *C. bombi* has not yet been fully elucidated. However, *C. bombi* is clearly diploid and screening shows an extreme diversity of genotypes in natural populations, to the extent that each host may carry its own infection 'cocktail' made up of almost unique strains (Schmid-Hempel and Reber Funk, 2004) (Salathé et al., Tognazzo et al., in preparation). The *Bombus–Crithidia* system has several advantages for the study of genetic exchange, such as accessible natural populations, the possibility to separate infections into single clones that can be grown in medium and genotyped, and the possibility to run experiments *in vitro* and *in vivo* in the laboratory or the field.

2. Materials and methods

2.1. Sampling and experimental design

Queens of *B. terrestris* freshly emerged from hibernation were collected in spring 2008 in Northeastern Switzerland and brought to the laboratory. At this point, faeces of all bees were taken and checked microscopically for cells of *C. bombi*. Faeces of infected queens were directly submitted to single cell sorting using fluorescence activated cell sorting (FACS) to separate cells and starting the cloning process (for detailed cloning methods, see Salathé et al., in preparation). The multilocus-genotype of one clone per host was determined using five available microsatellites (Schmid-Hempel and Reber Funk, 2004). Five clones with different multi-locus genotypes were selected for the infection experiment (Table 1), and labelled as strain A (corresponding to project tag 08.068), B (08.075), C (08.076), D (08.091), and E (08.161). The strains were chosen such that the specific allele combinations would make it more likely to see recombinants.

The uninfected queens were allowed to lay eggs and start a colony; then, six colonies were selected for the infection experiment. We labelled the colonies as C1 (corresponding to project tag 08.080), C2 (08.090), C3 (08.097), C4 (08.128), C5 (08.131), and C6 (08.184). Workers from these six colonies were not infected previously, and experimentally infected with either one ('singly infected') or two ('co-infected') clones of *C. bombi* resulting in a total of 15 possible combinations (five single and ten double infections). This infection scheme thus required 15 bees, and the scheme was repeated twice for all six colonies. Hence, there were 30 bees per colony, labelled by the colony prefix followed by a consecutive number (example: C1.15 is bee nr. 15 from colony C1); a total of 180 workers were infected. Bees were mildly starved (2 h) before the infection to encourage the complete take-up of the inoculum that was presented in 10 μl of sugar water (50% vol). For the inoculum, the two separately kept parental strains were mixed into an infection cocktail immediately (i.e. within minutes) before the 10 μl of sugar water was offered. The overall infection dose for each worker was set to 20,000 cells (i.e. for double infections: 10,000 cells per clone; for single infections: 20,000 cells for the clone). The same infection cocktail was used for several bees (from several colonies) on the same day. Not all bees could be infected on the same day and the same infection mixture (the infection cocktail) had to be prepared independently on several days. After infection workers were kept singly with pollen and nectar (Apiinvert® solution) *ad libitum*.

Seven days post infection faeces were sampled from each worker (following Schmid-Hempel and Schmid-Hempel, 1993) and checked microscopically for the presence of *C. bombi* cells. The faeces from infected workers were collected and submitted to single cell sorting by FACS between 10 and 20 days post-infection. Sorting resulted in single cells placed into 96 well PCR-plates (twin-tec PCR Plate 96, skirted; Eppendorf, Hamburg, Germany) containing 50 μl of culture media each. Plates were subsequently incubated for two weeks at 27 °C to ensure sufficient growth of the

Table 1
Genotypes of clones used in the infection experiment. Shown are the two alleles at the five tested loci. *C. bombi* is diploid.

Clone	Locus				
	Cri_4	Cri_2F10	Cri_4G9	Cri_16	Cri_1B6
A	125 131	115 117	138 154	119 122	142 148
B	129 133	117 125	150 154	116 119	138 142
C	129 131	117 117	138 138	116 122	150 158
D	131 133	117 125	154 158	116 122	150 160
E	133 133	117 119	144 150	119 119	136 152