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Global *Wolbachia* prevalence, titer fluctuations and their potential of causing cytoplasmic incompatibilities in tsetse flies and hybrids of *Glossina morsitans* subgroup species

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ABSTRACT

We demonstrate the high applicability of a novel VNTR-based (Variable-Number-Tandem-Repeat) molecular screening tool for fingerprinting *Wolbachia*-infections in tsetse flies. The VNTR-141 locus provides reliable and concise differentiation between *Wolbachia* strains deriving from *Glossina morsitans morsitans*, *Glossina morsitans centralis*, and *Glossina brevipalpis*. Moreover, we show that certain *Wolbachia*-infections in *Glossina* spp. are capable of escaping standard PCR screening methods by 'hiding' as low-titer infections below the detection threshold. By applying a highly sensitive PCR-blot technique to our *Glossina* specimen, we were able to enhance the symbiont detection limit substantially and, consequently, trace unequivocally *Wolbachia*-infections at high prevalence in laboratory-reared *G. swynnertoni* individuals. To our knowledge, *Wolbachia*-persistence was reported exclusively for field-collected samples, and at low prevalence only. Finally, we highlight the substantially higher *Wolbachia* titer levels found in hybrid *Glossina* compared to non-hybrid hosts and the possible impact of these titers on hybrid host fitness that potentially trigger incipient speciation in tsetse flies.

1. Introduction

Wolbachia are universal endosymbionts of most terrestrial arthropods and filarial nematodes that are mainly transmitted from mother to progeny and affect host biology in many ways. For example, these α-proteobacteria are capable of triggering a diverse repertoire of life-history traits in insects such as cytoplasmic incompatibility (CI), sex ratio distortion, longevity, innate immunity, locomotion, olfaction, toxin-sensitivity as well as sexual mating behavior changes (recently reviewed in Schneider et al. (2010)). *Wolbachia* are, next to the γ-proteobacteria *Sodalis glossinidius* and *Wigglesworthia glossinida*, part of the triple-symbiont association present in tsetse flies (reviewed in Aksoy and Rio (2005)). Over the last years, numerous studies have focused on the complex interactions exhibited between tsetse flies and their symbionts (Aksoy and Rio, 2005). This extensive research is of great value and interest not solely for the symbiosis research community, but also for medicine-related fields as tsetse are key vectors of disease. Within the genus *Trypanosoma* (*Kinetoplastida*), particularly the species *T. brucei*, *T. congolense* and *T. vivax* are important disease causation parasites. The sub-species *T. brucei rhodesiense* (*Tbr*) and *T. brucei gambiense* (*Tbg*) are the causative agents of Human African Trypanosomiasis (HAT or sleeping sickness). *T. b. brucei* (*Tbb*), *T. congolense* (*Tg*) and *T. vivax* (*Tv*) cause Animal African Trypanosomiasis (AAT or Nagana), primarily in cattle. Of the 32 tsetse fly species all tested species have been shown experimentally to be able to transmit trypanosomes, but only nine of these, belonging to the *Glossina palpalis* and *Glossina morsitans* groups, normally transmit sleeping sickness. Members of the *palpalis* group (*G. palpalis*, *Glossina fuscipes*, and *Glossina tachinoides*) are the main vectors for *Tbg*, whereas *Tbr* is mostly transmitted by species of the *morsitans* group (*G. morsitans*, *Glossina pallidipes*; www.who.int). According to the World Health Organization (WHO), 23 out of 25 sub-Saharan countries in Africa were reported HAT-infested between 2000 and 2009 (Simarro et al., 2010). Currently, no vaccines against sleeping sickness are available and treatment options are generally very limited. Thus, novel strategies for fighting this health burden are urgently required. So-called biological pest control strategies, targeting the vector biology, have become highly attractive. One such strategy is the classic sterile insect technique (SIT) that relies on eradication of insect populations by releasing irradiation-generated sterile males into the field (Knipling, 1955). Lately, alternatives to the aforementioned method based on targeting host–symbiont interrelation, are being investigated.

Wolbachia-infections in insects were shown to cause reproductive phenotypes such as cytoplasmic incompatibility CI, function-

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ing as a post-mating barrier to hybrid formation (recently reviewed in Saridaki and Bourtzis (2010)). CI results in high levels of embryonic lethality among the offspring and can be either uni- or bidirectional. Unidirectional CI occurs when *Wolbachia*-uninfected females mate with infected males; bidirectional CI arises in mates where both partners harbor different, incompatible *Wolbachia*-infections (recently reviewed in Merçot and Poinsot (2009)). Hence, *Wolbachia*-induced CI could be exploited to induce natural reproductive sterility in tsetse fly populations and consequently hinder the transmission of *Trypanosoma* (Sinkins and Gould, 2006; Rasgon, 2008; Brelsfoard and Dobson, 2009). This idea, which was already discussed in the 1940s (Potts, 1944; Vanderplank, 1944), is currently revived, particularly with respect to a very recent study. Alam et al. (2011) have reported on the expression of strong unidirectional CI in crosses of *Wolbachia*-infected *Glossina morsitans morsitans* to antibiotic-treated ones, pinpointing the biological significance of these symbionts in tsetse flies (Alam et al., 2011). These recent data suggest that tsetse fly *Wolbachia* can cause CI, perhaps not only in *G. morsitans morsitans* hosts but also in other *Glossina* species.

During the past decades, hybridization experiments have been conducted between various members of the genus *Glossina*, producing female hybrids with reduced fecundity and sterile male hybrids under laboratory conditions (reviewed in Gooding (1990)). Moreover, natural hybridization events between *Glossina* species in the field have been reported repeatedly, suggesting that pre-mating barriers to hybrid formation are rather weak between sympatric members of this genus (Corson, 1932; Potts, 1944; Vanderplank, 1944, 1947, 1948; Gooding, 1993). Extensive studies on mating behavior have uncovered that *Glossina swynnertoni* females, for example, are not very choosy and accept all mates regardless of whether they are con-specific or not (Gooding, 1993). *G. swynnertoni* males attempt to mate equally with females of *G. swynnertoni*, *G. m. morsitans* and *G. m. centralis*, regardless of con-specificity of the mates (Gooding, 1993) and *G. swynnertoni* males are able to both inseminate and fertilize *G. m. morsitans* females (Gooding, 1993, 1997). Hence in the evolutionary point of view the *G. morsitans* group is considered a very young and highly dynamic species complex with weak pre-mating isolation but significant post-mating barriers by expressing high hybrid mortality and complete hybrid male sterility (Gooding, 1999).

Since unidirectional incompatibilities were observed in crosses of certain *G. morsitans* sub-species, where one crossing direction was less compatible than the reciprocal one (Curtis, 1972), the basis of such mating incompatibilities has been attributed to maternally inherited cytoplasmic factors (Gooding, 1987). Hence, with the discovery of *Wolbachia* in tsetse fly ovaries (O'Neill et al., 1993), the authors speculated that this well-known reproductive parasite might act as the causative agent for triggering post-mating isolation between tsetse fly species in nature. However, for acting as a true "speciation factor", high prevalence and transmission frequency of maternally-transmitted *Wolbachia* are indispensable in order to avoid gene flow between emerging species (Coyne and Orr, 2004). Therefore it is of pivotal interest to thoroughly assign native *Wolbachia* infection frequencies throughout the genus *Glossina* from the field as well as from lab colonies.

Two recent studies have demonstrated the patchy distribution of *Wolbachia* in *Glossina* spp. Cheng et al. (2000) showed that infections among *G. swynnertoni* from field samples exhibited 11% *Wolbachia*-infection rate (Cheng et al., 2000), whereas *G. austeni* range from 0% to 98% and from 0% to 30% in *G. brevipalpis*. The most recent study on *Wolbachia*-prevalence within the genus *Glossina* came up with infection rates ranging from 0% to 100% in the *morsitans* group, from 0% to 8% in the *palpalis* group, and from 2% to 40% within the *fusca* group (Doudoumis et al., 2012; see Fig. 1 for *Glossina* phylogeny). Regarding the patchy *Wolbachia*-distribution demonstrated in both field and laboratory sampling sets of *Glossina* spp. (Cheng et al., 2000; Doudoumis et al., 2012), the question arises, whether these findings might be influenced by the inefficiency of standard molecular screening tools that do not detect *Wolbachia* low-titer infections. Indeed, detection of low-titer infections and reliable strain typing of closely related *Wolbachia* symbionts in insects has proven challenging and is dependent on the choice and information value of marker genes under consideration. Historically, the main body of *Wolbachia* strain-typing approaches and phylogenies were elaborated on the basis of sequence data derived from the 644-bp sequence of the highly dynamic *Wolbachia* Surface Protein gene *wsp* (Zhou et al., 1998), which is under strong adaptive evolution and a hotspot for inter-strain recombination (see Werren and Bartos, 2001). However, the *wsp* gene is not informative with respect to distinguish CI-inducing from neutral or even mutualistic strain phenotypes (Iturbe-Ormaetxe et al., 2005). Recently, *Wolbachia*-specific Multi Locus Strain Typing marker systems (MLSTs) were successfully used for phylogenetic strain typing (Baldo et al., 2006; Paraskevopoulos et al., 2006). For higher resolution of strain phylogenies and to distinguish even very closely related *Wolbachia* strains in different host systems we have recently developed new sets of hyper-variable marker systems covering mobile insertion sequences (IS elements) and Variable-Number-Tandem-Repeat (VNTR) loci (Riegler et al., 2005, 2012; Miller and Riegler, 2006). As shown earlier, multiple-locus VNTR analysis (MLVA) is a highly successful method for studying genetic variability of many bacterial species, that was originally introduced for molecular typing of pathogens like *Bacillus anthracis* (Keim et al., 2000; Klevytska et al., 2001; Liao et al., 2006; and reviewed in Top et al. (2004) and Lindstedt (2005)). Indeed, VNTRs have been proven to provide a high level of discriminatory power for strain differentiation because of their high susceptibility to mutation by replication slippage and ectopic recombination between cluster units (van Belkum et al., 1998). Based on the complete genome sequence of *Wolbachia* from *D. melanogaster* wMel (accession number

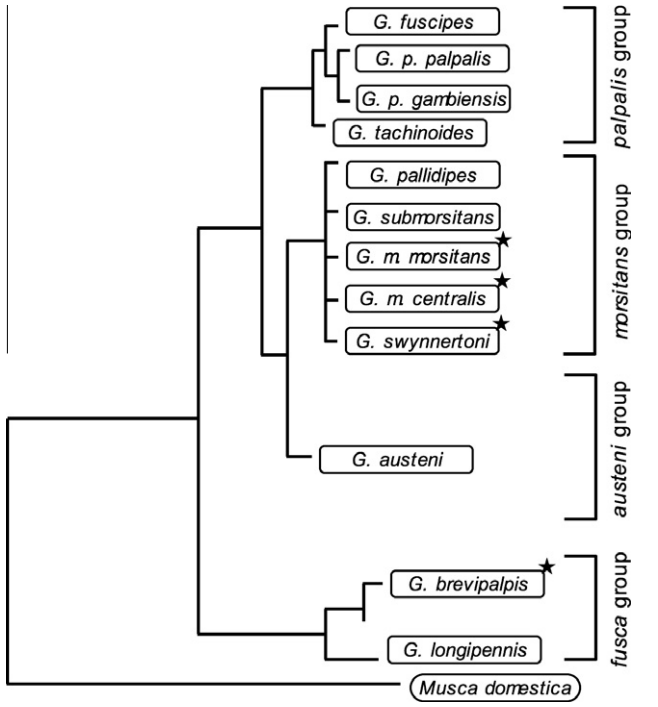

Fig. 1. *Glossina* phylogeny. The phylogenetic tree of the genus *Glossina* is based on IST-2 sequence data and was adapted from Chen et al. (1999). The figure depicts four groups of *Glossina* spp.: *palpalis*, *morsitans*, *austeni*, and *fusca*. Tsetse flies from the *morsitans* (*G. m. morsitans*, *G. m. centralis*, *G. swynnertoni*) and the *fusca* group (*G. brevipalpis*) were analyzed in this study (indicated by asterisks).