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Phylogenetically distinct *Wolbachia* gene and pseudogene sequences obtained from the African onchocerciasis vector *Simulium squamosum* ☆

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ABSTRACT

Wolbachia are intracellular bacteria mostly found in a diverse range of arthropods and filarial nematodes. They have been classified into seven distinct 'supergroups' and other lineages on the basis of molecular phylogenetics. The arthropod-infecting *Wolbachia* are usually regarded as reproductive parasites because they manipulate their host species' sexing system to enhance their own spread, and this has led to their investigation as potential agents of genetic control in medical entomology. We report 12 partial *Wolbachia* gene sequences from: *aspC*, *aspS*, *dnaA*, *fbpA*, *ftsZ*, *GroEL*, *hcpA*, *IDA*, *rpoB*, *rpe*, *TopI* and *wsp* as well as a single *ftsZ* pseudogene sequence, which have all been PCR-amplified from *Simulium squamosum* (Diptera: Simuliidae). To our knowledge this is the first such report from Simuliidae. Uninterrupted open-reading frame sequences were obtained from all 12 genes, covering ~6.2 kb of unique DNA sequence. Phylogenetic analyses with the different coding genes gave consistent results suggesting that the *Wolbachia* sequences obtained here do not derive from any of the known *Wolbachia* supergroups or lineages. Consistent with a unique genetic status for the *S. squamosum Wolbachia*, the hypervariable regions of the *Wolbachia*-specific *wsp* gene were distinct from all previous records in both sequence and length. As well as potential implications for newly emerging *Wolbachia*-based disease control methods, the results may be relevant to some problems experienced in the laboratory colonisation of *Simulium damnosum* sensu lato and why it is such a diverse species complex.

1. Introduction

Wolbachia are maternally inherited intracellular bacteria commonly found in the reproductive tissues of certain arthropods and nematodes. There is a single species, *Wolbachia pipientis*, described by Hertig in 1936. Although the *Wolbachia* strains found in nematodes are quite diverse and are represented in three *Wolbachia* supergroups (phylogenetically distinct molecular clades), their known host range is mostly limited to filarial worms of the family Onchocercidae (Fenn et al., 2006; Haegeman et al., 2009). Arthropods support both a wider diversity of *Wolbachia* and provide a broader range of host species. There are three major arthropod taxa known to host *Wolbachia* including insects, crustacea and arachnids (Duron et al., 2008; Ros et al., 2009) and of these three, insects appear to be the most competent hosts, with an estimated 20–70% of species being infected (Werren et al., 1995; Jeyaprakash and Hoy, 2000; Bourtzis, 2008).

There is no single gene which has been sequenced from at least one member of all the *Wolbachia* supergroups; therefore to reliably classify a new *Wolbachia* strain it is necessary to obtain at least two gene sequences. All of the supergroups are represented with at least one *ftsZ* gene sequence (except G and J), and at least one *GroEL* gene sequence (except G and E) (Ros et al., 2009). The G supergroup was described on the basis of the *wsp* gene, but its uniqueness seems to be the result of intragenic recombination between *wsp* genes from supergroups A and B, whereas other genes place members of G securely within supergroup A. The *wsp* gene is therefore considered unsuitable for phylogenetic estimation and supergroup G is considered to be an artefact (Baldo and Werren, 2007). It follows that *GroEL* and *ftsZ* genes should be sufficient to assign a novel *Wolbachia* strain (Rowley et al., 2004; Ros et al., 2009). However, supergroups I, J and K each contain *Wolbachia* from a single host species, and whilst current evidence indicates that they are distinct lineages (Ros et al., 2009), it is considered prudent not to assume supergroup status until this is confirmed by further data (Bordenstein et al., 2009). Due to these sorts of difficulties in

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under Accession Nos. FN563970–FN563982.

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confidently making supergroup designations, a Multi-Locus Sequence Typing (MLST) system was proposed for *Wolbachia* (Baldo et al., 2006; Baldo and Werren, 2007). This system initially used five genes to fulfil particular requisites, but Bordenstein et al. (2009) have recently expanded it to include 21 genes. A large dataset now exists for MLST analyses and it is therefore possible, by sequencing a subset of genes, to more accurately infer the relationship between a novel *Wolbachia* strain with that of the currently accepted supergroups (A–F and H) and lineages, than can be achieved with a single gene alone.

The phylogenetic evolution of the *Wolbachia* of filarial nematodes appears to follow that of the host taxa which has been taken to mean that their long-term evolutionary transmission is tied to the success of their host (Casiraghi et al., 2005). When filarial worms are treated with antibiotics, death of the *Wolbachia* results in the death of the worm. Filarial *Wolbachia* are thus regarded as mutualists that are required for the proper moulting and sexual development of their filarial host, and for this reason have become the focus of much research for drug design (Fenn et al., 2006). The *Wolbachia* that affect arthropods seem to operate in an entirely different way (see below), but they have also become a focus of disease control interest (Bourtzis, 2008; Cook et al., 2008; Rasgon, 2008). In most cases antibiotic 'curing' of arthropod *Wolbachia* infections does not prevent normal development, and in the rare cases where this does happen (Pannebakker et al., 2007) it has been described as a genetic 'addiction' rather than a case of mutualism (Werren et al., 2008). Certainly, there are far fewer clear cases of long-term vertical transmission of arthropod *Wolbachia* and far more examples of horizontal transfer which, taken together, suggest that the arthropod *Wolbachia* are reproductive parasites spreading at the evolutionary expense of their host (Fenn et al., 2006; Werren et al., 2008). They operate by manipulating their host sexing systems in order to spread. The most common mechanism is cytoplasmic incompatability, whereby females which are already infected gain a reproductive advantage (thereby passing on their infected cytoplasm) because they can mate successfully with both infected and uninfected males, whereas eggs from uninfected females are sterile if that female has mated with an infected male. Other mechanisms include male killing, feminisation of genetic males and thelytokous parthenogenesis (Werren et al., 2008). The potential for using *Wolbachia*-induced cytoplasmic incompatability as a genetic drive mechanism to force disease-refractory genes through vector populations has long been recognised and modelled (Curtis, 1992; Curtis and Sinkins, 1998; Brownstein et al., 2003; Rasgon et al., 2003), and recently this theory has come a step closer to a practical reality with the stable transformation of the dengue vector *Aedes aegypti* with the life-shortening *Wolbachia* *w*MelPop isolated from *Drosophila melanogaster* (McMeniman et al., 2009).

In humans, onchocerciasis is a severely debilitating tropical disease which results from infection by the filarial nematode *Onchocerca volvulus*. Approximately 36 million people are infected (Loewenberg, 2008), mostly in Africa, resulting in the annual loss of approximately one million disability adjusted life years (Boatin and Richards, 2006; Traoré et al., 2009). Symptoms, including blindness and skin disease, seem to result from a host reaction to *Onchocerca*-derived *Wolbachia* rather than to the filaria itself (Saint André et al., 2002). The World Health Organisation (WHO) African Programme for Onchocerciasis Control is controlling the disease through community directed treatment with the microfilaricidal drug ivermectin (Taylor et al., 2009). However, there is the danger of ivermectin resistance evolving (Osei-Atweneboano et al., 2007), and it is not clear whether ivermectin alone can eliminate transmission of the parasite in an African context (Borsboom et al., 2003). For these reasons new drugs are being sought and amongst the most promising targets are the *Onchocerca Wolbachia*, because the use of antibiotics to kill the *Wolbachia* consequently results in the death of the worm.

Onchocerciasis is transmitted by blood-sucking flies of the family Simuliidae (blackflies) and 95% of onchocerciasis is transmitted by sibling species of the *Simulium damnosum* complex (Post et al., 2007). The *S. damnosum* complex consists of 55 distinct cytoforms (Post et al., 2007) making it the largest known species complex of any insect. The different sibling species differ in many ways which affect the transmission of onchocerciasis. For example, some species do not bite humans and others do bite humans and take up the parasite, but the parasite does not develop (Post et al., 2007).

There are no published records of *Wolbachia* infecting Simuliidae but if they were infected there are aspects of their biology and their role in onchocerciasis transmission and control that could be affected. For example, it is already clear that *Wolbachia* from quite diverse species can be stably introduced into non-infected host species, but it is also clear that there are insect species that are resistant to one or more types of *Wolbachia* (Werren et al., 2008). For example, despite some considerable effort, *Wolbachia* has never been stably introduced into a malaria vector (*Anopheles* mosquito) population (Jin et al., 2009). The presence of *Wolbachia* in *S. damnosum* sensu lato would demonstrate that its cytoplasm can host the bacteria, and that it may be suitable for *Wolbachia*-based control methods such as those proposed for *A. aegpyti* (Ruang-areerate and Kittayapong, 2006) and other insects (Bourtzis, 2008; Cook et al., 2008). Most insect *Wolbachia* reproductively parasitise their host by cytoplasmic incompatibility, which prevents non-infected females producing offspring with an infected male (Werren et al., 2008). There have been clear experiments where antibiotics have been used to cure closely related *Wolbachia*, which has allowed the formation of viable host hybrids, that do not occur in nature, and thus it seems that *Wolbachia* represented the major genetic barrier between these species. Therefore, *Wolbachia* are widely recognised to have a role in arthropod speciation (Bordenstein et al., 2001; Werren et al., 2008) and could have promoted the abundance of sibling species in the *S. damnosum* complex. Because these different cytoforms vary in their ability to host *O. volvulus* (see above), it would be interesting to explore the taxonomic relationship between the *Onchocerca Wolbachia* and any possible vector *Wolbachia*. One of the main signs of arthropod *Wolbachia* is sex-ratio distortion and there is some indication of this in the *S. damnosum* complex. On average, samples of larvae prepared for cytotaxonomy contain slightly more females than males, but this is probably largely or completely explained by the known faster development of males. Only one cytospecies of the *S. damnosum* complex has been colonised in the laboratory (the Beffa form of *Simulium soubrense*), and this has only been achieved three times. On the first occasion the colony sex ratio became progressively distorted in favour of females until the colony died out in the sixth generation (Simmons and Edman, 1982). On the other two occasions (Raybould and Boakye, 1986) the sex ratio was possibly distorted in favour of females in one colony and significantly distorted in favour of males in the other, but both colonies died out due to poor survival of larvae and adults.

2. Materials and methods

2.1. Host collection, identification and DNA extraction

Simulium damnosum sensu lato larvae were collected in Ghana, between 2006 and 2008, from the River Pawnpawn at Boti Falls (6°10′N 0°11′W). Specimens were preserved and identified (to the species and cytospecies level) using the methods described by Crainey et al. (2009). All specimens used in this study were identified as *Simulium squamosum*. One DNA preparation was made