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Chromosomal localization of *Wolbachia* inserts in the genomes of two subspecies of *Chorthippus parallelus* forming a Pyrenean hybrid zone

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Abstract

Wolbachia are endosymbiotic bacteria of arthropods and nematodes that can manipulate the reproduction of various host organisms to facilitate their own maternal transmission. Moreover, *Wolbachia*'s presence in host germ cells may contribute to the many cases of lateral gene transfer from *Wolbachia* to host genomes that have been described. A previous study in *Chorthippus parallelus*, a well-known orthopteroid forming a hybrid zone in the Pyrenees, identified *Wolbachia* sequences from two major supergroups in the genomes of infected and uninfected *Chorthippus parallelus parallelus* (*Cpp*) and *Chorthippus parallelus erythropus* (*Cpe*) subspecies. In this study, we map the *Wolbachia* genomic inserts to specific regions on the chromosomes of *Cpp* and *Cpe* by Fluorescent *in situ* Hybridization (FISH) using tyramides to increase the accuracy and detection of these insertions. Additionally, we consider some of the possible roles that these bacterial inserts play in the organization and function of the grasshopper genome, as well as how they can serve as markers for phylogenetic relationships of these organisms.

Keywords

Wolbachia; Horizontal gene transfer; Bacterial chromosome insertions; Hybrid zone; *Chorthippus parallelus*; FISH; Tyramides

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INTRODUCTION

Chorthippus parallelus (*Cp*) is an orthopteroid insect of the family Acrididae (Zetterstedt 1821), which forms a hybrid zone in the Pyrenees where two of its subspecies, *C. p. parallelus* (*Cpp*) and *C. p. erythropus* (*Cpe*), meet. *Cpp* is widely distributed throughout the European continent, while *Cpe* is endemic to the Iberian Peninsula. It is estimated that the hybrid zone of *Cp* originated approximately 9,000 years ago (Hewitt 1988; Hewitt 2001) from two formerly allopatric subspecies during the last ice age (Hewitt, 1996; Gomez and Lunt 2007; Hewitt 1999, 2011). While F1 hybrid males produced in the laboratory are sterile in accordance with Haldane's rule (Hewitt et al. 1987; Bella et al. 1990), hybrids inhabiting the contact zones are generally fit, viable and fertile after generations of coadaptation of genes from both pure species within a hybrid genome. Several studies have defined morphological, ethological, chromosomal, and molecular differences between the two subspecies that are used as markers of each *Cp* subspecies and their hybrids (Butlin and Hewitt 1985a, b; Gosálvez et al. 1988; Ritchie 1990; Cooper et al. 1995; Lunt et al. 1998; Ibrahim et al. 2002; Bella et al. 2007; see Shuker et al. 2005 for a review). Of all the reported markers, the cytogenetic X-linked ones are those that show the most striking differences between *Cpp* and *Cpe* subspecies: the distribution and/or composition of the heterochromatic regions differ between the *Cpp* and *Cpe* X chromosomes, including an extra Nucleolar Organizing Region (NOR) present only in *Cpp* (Gosálvez et al. 1988; Serrano et al. 1996; Bella et al. 1993, 2007).

The endosymbiont *Wolbachia* sp. (Rickettsiales) is a genus of obligate, intracellular alpha-proteobacteria that infects 40–52% of terrestrial arthropod species, as well as certain nematode species (Roman and Hammerstein 2012; Zug and Hammerstein 2012; Weinert et al. 2015). Generally, it has been classified as a single species, *W. pipientis*, with unique strains that form phylogenetically divergent clusters designated as supergroups (Werren 1997; Jeyapragash and Hoy 2000; Bordenstein et al. 2009, Lindsey et al. 2016). For example, phylogenetic analyses of *Wolbachia* 16S rRNA gene in *Cp* demonstrated the existence of at least two different *Wolbachia* supergroups (B and F) infecting this grasshopper (Bella et al. 2010; Zabal-Aguirre et al. 2010).

The importance of *Wolbachia* in evolutionary biology is due in part to its ability to act as a reproductive isolation barrier (Bordenstein et al. 2001; Jaenike et al. 2006; Miller et al. 2010; Shropshire and Bordenstein 2016) and an evolutionary agent that can change host sex ratios and sex determination mechanisms (Wade 2001; Charlat et al. 2003, Werren et al. 2008). Such alterations span feminization of males, male killing, induction of parthenogenesis in non-parthenogenetic species, and cytoplasmic incompatibility (CI). CI results in embryonic lethality of offspring from an infected male mated to either an uninfected female or an infected female with a different *Wolbachia* strain and is the most common host modification produced by *Wolbachia* (reviewed in Werren et al. 2008; Serbus et al. 2008; LePage and Bordenstein 2013). CI-inducing *Wolbachia* present in both subspecies of *Cp* may contribute to the evolutionary dynamics of the hybrid zone by preventing hybridization of grasshoppers with incompatible *Wolbachia* strains (Zabal-Aguirre et al. 2010, Zabal-Aguirre et al. 2014). Furthermore, a previous study found that *Wolbachia* negatively affects chiasmata formation and induces spermatid malformation in hybrid males of this species; these cytogenetical

effects may result in modifications of paternal chromatin that contribute to the pathology of CI (Sarasa et al. 2013).

Though exclusively transmitted through the maternal line, *Wolbachia* infect the sexual organs of both male and female arthropods, including the germline stem cells and stem cell niches (Frydman et al. 2006; Fast et al. 2011; Toomey et al. 2006; Toomey and Frydman 2014). As intracellular bacteria in such close proximity to the germline genome, it is not surprising that many cases of gene transfer from *Wolbachia* to its host have occurred (Aikawa et al. 2009; Kondo et al. 2002; Fenn et al. 2006; Bordenstein 2007; Nikoh et al. 2008; Klasson et al. 2009; Dunning Hotopp 2011; Brelsfoard et al. 2014; Klasson et al. 2014; Funkhouser-Jones et al. 2015). Some of the inserts are of considerable size or quantity (>100 kb) (Aikawa et al. 2009; Kondo et al. 2002; Nikoh et al. 2008; Funkhouser-Jones et al. 2015) and, in some cases, more than one insertion is present in the host genome (Dunning Hotopp 2011; Brelsfoard et al. 2014; Klasson et al. 2014; Funkhouser-Jones et al. 2015). We recently reported the first evidence for gene transfer from two discrete *Wolbachia* supergroups (B and F) into the nuclear genome of *Cp*, revealing that some inserts are subspecies-specific while others are present in both subspecies (Funkhouser-Jones et al. 2015). Here, we use FISH to cytogenetically localize and analyze these inserts in the chromosomes of both subspecies and discuss their possible origin in light of the evolutionary history of this organism and their contribution to its current genome organization.

MATERIAL AND METHODS

Sample collection

Male adult specimens of *Cpp* and *Cpe* were collected from the pure populations of Gabas (France) and Escarrilla (Spain), respectively, in both ends of the hybrid zone in the Pyrenees Mountains (Zabal-Aguirre *et al.* 2010). Grasshopper gonads were extracted and fixed in fresh absolute ethanol: glacial acetic acid (3:1) to be used to prepare slides, and the rest of the body was fixed in absolute ethanol for further DNA isolation. All materials were kept at −20°C until use.

DNA isolation and PCR identification of uninfected individuals

DNA extraction was carried out by the standard phenol-chloroform method (Sambrook et al. 1989). PCR amplifications were performed to analyze *Wolbachia* infection in 50 *Cp* specimens using the following *Wolbachia* 16S rRNA gene specific primers: 16SF 5'-ACTGCTACCTTGTTACGACTT-3', 16SR 5'-TTGTAGCTTGCTATGGTATAACT-3' (Zabal-Aguirre et al. 2010). PCR products were sequenced and homology searches were performed at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Generation of FISH probes

Three *Wolbachia* contigs from previous genome sequencing of uninfected *Cpp* grasshoppers (Funkhouser-Jones et al. 2015) were amplified using PCR in uninfected individuals. Cpar-Wb1 and its primers were previously described in Funkhouser-Jones et al. 2015. The following primers were designed to amplify Cpar-Wb2 and Cpar-Wb3: 1882contigF (5'-

TCAGGGAGATCGAGTCAAGG-3'), and 1882contigR (5'-GGAAGACGATGATGGGTTTG-3') for Cpar-Wb2 and 203contigF (5'-GCAGCTGAGGCTTATCTTGG-3'), 203contigR (5'-CCAATGCCGAAAACCTTCAT-3') for Cpar-Wb3. PCR reactions were performed in 1X Buffer, 2 mM MgCl₂, 2 μM dNTPs (Roche), 1.2 μM of each primer, 1.25 U of BIOTAQ DNA polymerase (Biotools), and 100 ng of genomic DNA. The PCR program started with a denaturation of 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at 56 °C and 3 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were run on 0.7% TAE agarose gels and purified using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare).

The purified PCR products were used to generate FISH probes with the DecaLabel DNA Labeling kit (Thermo Scientific), which is based on the random-primer method (Feinberg and Vogelstein 1983; 1984). The complete reaction was a modification of the kit protocol and consisted of 10 μl of decanucleotide, 5X Reaction Buffer, 1 μg of cDNA, and nuclease-free H₂O to a final volume of 42 μl incubated at 100 °C for 10 min. We then added 1 mM dNTPs mix, 1.75 μl of Digoxigenin-11-dUTP (Roche), and 1 μl of Klenow enzyme and incubated at 30 °C for 2 hours. The probes were purified with the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare), resuspended in 50 μl of H₂O, and stored at -20 °C until further use.

Tyramide-coupled FISH

Chromosome slides were prepared from fixed male gonads to observe results over meiotic chromosomes in the larger stage of bivalents, diakinesis. Slides were prepared by the conventional technique of squashing, and the coverslip was removed after immersing the slides in liquid nitrogen. The squashed biological material was treated for 5 min with pepsin (50 μg/ml in 0.01 N HCl) at 37°C, followed by a 30 min incubation in 2% paraformaldehyde at room temperature. FISH coupled with tyramide amplification (FISH-TSA) was performed according to Krylov et al. 2007 and 2008, with the following minor changes: endogenous peroxidases were inactivated in 1% H₂O₂ for 30 min. After that, slides were dehydrated in a series of methanol washes (70%, 85%, 100%) and allowed to dry.

Chromosomes were denatured and hybridized with 50 μl of hybridization mixture under a cover glass for 5 min at 70°C. The hybridization mixture was composed of 2 μl of the labeled probe, 50% formamide, and 2X SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0). Slides were then left overnight in a wet chamber at 37°C. Posthybridization washing and visualization of FISH-TSA signal were also performed as described by Krylov et al. 2007 and 2008. Detection of probes with Antidigoxigenin-POD (Roche) was performed at a concentration of 1:2000 in blocking buffer (TNB-0.1 M Tris/HCl, 0.15 M NaCl, 0.5% Blocking reagent). The tyramide solution (Perkin Elmer) was incubated onto the slides for 5 min at a concentration of 1:50. Chromosomes were counterstained with 50 ng/μl of DAPI (4',6-diamidino-2-phenylindole, Roche) diluted in Vectashield (Vector Laboratories). Results were observed in a digital image analysis platform based on Leica DMLB fluorescence microscope with two independent green and blue filters. Images were captured as tiff files using a cooled CCD Leica DF35 monochrome camera (Leica Microsystem) and

final images were edited using Photoshop CS6 (Adobe). Six individuals from each subspecies and a minimum of 20 diakinesis stages per individual were analyzed.

C-Banding

The C-Banding technique was performed following the conventional protocol: 10 min incubation with 5% Ba(OH)₂ (diluted in H₂O), 5 min in H₂O with 2 drops of acetic acid, and 30 min in 2X SSC at 60 °C, as described in Bella et al. (1993). After washing thoroughly, the C-bands were stained with propidium iodide (25 µg/ml diluted in 1X PBS) under a cover glass for 30 min. Finally, the slides were washed with 1X PBS, and mounted with Vectashield (Vector Laboratories). Results were observed and images captured employing the same platforms described above.

RESULTS

1) *Wolbachia* sequences are integrated into the genomes of both *Cp* subspecies

Our previous genomic study of *Wolbachia*-to-grasshopper gene transfer identified over 650 kb of *Wolbachia* DNA in the genome of uninfected *Cpp*, while cytological staining of *Cp* chromosomes verified that a 1.8 kb *Wolbachia* contig, Cpar-Wb1, was integrated into both the *Cpp* and *Cpe* genomes (Funkhouser-Jones *et al.* 2015). Here, positive PCR amplifications of the Cpar-Wb1 contig and two additional contigs, Cpar-Wb2 and Cpar-Wb3 (each 3kb in length, Table S1), in uninfected *Cpp* and *Cpe* grasshoppers confirm the integration of multiple *Wolbachia* sequences in the genomes of both subspecies of *Cp*

2) Tyramide-coupled FISH analysis corroborates a widespread representation of these three *Wolbachia* inserts in *Cp* chromosomes

Tyramide-coupled FISH analysis shows a widespread distribution of the three *Wolbachia* contigs in the *Cpp* and *Cpe* karyotypes (Figure 1, but see also Funkhouser-Jones et al. 2015 figures for complementary information). These data corroborate the PCR results and localize them to specific chromosomal locations. The FISH patterns reveal location similarities in both subspecies but also striking differences between *Cpp* and *Cpe*. It is remarkable that the majority of the hybridization signals in both subspecies are located in the telomeric regions, although interstitial signals in L1, M4, M5, and X chromosomes are also present (Figure 1). To properly identify the *Cp* chromosomes, C-banding technique was carried out after the tyramide-coupled FISH in *Cpe* and *Cpp* (Figure 2). Since C-banding reveals specific heterochromatic regions on each bivalent, it permits exact identification of chromosomes in both subspecies that are difficult to differentiate, such as chromosomes L1 and L2. It is of note that the X chromosome in the *Cpe* subspecies contains centromeric and interstitial constitutive heterochromatic regions, while the constitutive heterochromatin appears in centromeric and telomeric regions in the *Cpp* X chromosome. Nevertheless, the L2 and L3 chromosomes have the same constitutive heterochromatic pattern in both *Cp* subspecies: interstitial and centromeric heterochromatin in L2, and telomeric and centromeric in L3 (Figure 2), as previously described by Gosálvez et al. (1988), Bella et al. (1993; 2007) and Serrano et al. (1996).

3) The integrated sequences of *Wolbachia* tend to accumulate in the constitutive heterochromatin of *Cp*

All information obtained with the tyramide-coupled FISH assays is summarized in Figure 3. Interestingly, there are several positions that show the three inserts in the same location, not only within the same subspecies, but also in both subspecies (for example the telomeric position in M4, M6, S7 and S8 chromosomes). In contrast, there are some chromosomes with a unique insert localization that is subspecies-specific (such as the interstitial localization of the Cpar-Wb2 insertion in the longer arm of L1 and L2 *Cpp* chromosomes). Although inserts show a clear tendency to accumulate in the constitutive heterochromatin, not all of the inserts' signals appear in such regions, and not all the constitutive heterochromatic areas have insertions. It is noteworthy that the centromeres do not show insertions of any type, as opposed to the telomeric regions (Figure 3). Also of note is the different distribution patterns of these sequences in the X chromosomes. For example, in *Cpe*, the inserts localize together at the heterochromatic interstitial band of the X chromosome, which is not found in *Cpp*, even though the Cpar-Wb1 insert localizes in a nonheterochromatic interstitial position in both *Cp* X chromosomes. Also, in *Cpp* X chromosomes, we observe an irregular dotted signal for the Cpar-Wb2 insert and a telomeric signal for the Cpar-Wb3 sequence.

DISCUSSION

PCR amplification of the contigs used in this study in *Cp* individuals, regardless of infection status, corroborates our previous findings that the sequences are not false positives due to living *Wolbachia* or contamination (Funkhouser-Jones *et al.* 2015). This is also demonstrated by chromosomal FISH since the absence of non-specific hybridization signal (dispersed dots) either in the cytoplasm or on the chromosomes rules out a cytoplasmic *Wolbachia* infection.

The FISH approach with tyramides permits the physical mapping of the contigs since genomic studies can detect the insertions but do not show the distribution and localization of these sequences. The results here provide further information on i) the biology of *Wolbachia* and its frequent lateral transfer to its host, ii) the evolutionary history of the subspecies, and iii) tyramide-coupled FISH as a tool for further studies and models since this sensitive methodology reveals the position of small contigs of any source in a given karyotype. However, it is important to point out that the size or intensity of the FISH signals observed do not necessarily correlate with the number, size or orientation of the sequences detected, but do clearly indicate their presence.

Our previous phylogenetic analyses revealed that *Wolbachia* inserts in the *Cp* genome probably originated from relatively recent independent transfers from both B and F *Wolbachia* supergroups (Funkhouser-Jones *et al.* 2015). Though recombinant *Wolbachia* have been detected in the hybrid zone formed by these grasshoppers (Martinez-Rodriguez *et al.* 2016), it is unlikely that the insertions arose from a recombinant or “hybrid” B and F *Wolbachia* strain with genes from both supergroups transferring in a single event into the *Cp* genome (Funkhouser-Jones *et al.* 2015) due to the extensive number of redundant genes from both the B and F *Wolbachia* in the grasshopper nuclear genome (Funkhouser-Jones *et*

al. 2015). The role that these bacterial sequences play in the organization and function of the grasshopper's genome is uncertain. Altogether, the two *Wolbachia* supergroups (B and F) transferred at least 448 kb and 144 kb of DNA, respectively, into the host nuclear genome (Funkhouser-Jones et al. 2015). The contribution of these *Wolbachia* insertions represents around 4% of the global *Cp* genome, which is not negligible considering the giant size of the grasshopper genome: around 14.1 Gb (Lechner et al. 2013; R. Pereira, com. per.). However, our previous analysis did not allow us to distinguish whether these were large insertions or multiple smaller inserts spread throughout the genome. Our current results provide information on the physical mapping of these inserts and on the genomic and nuclear architecture of *Cp* (Figure 3). In absence of information of the expression of these inserts and given their preferential location in previously described C-heterochromatic regions [similar to *Wolbachia* insertions in the *Drosophila ananassae* genome (Klasson et al. 2014)], we presume that most of them probably do not play a concrete role in grasshopper biology, unless they interrupt a coding sequence or interfere in a gene's expression.

The location of the fluorescent signals reported here are consistent across all individuals analyzed. This makes false positives unlikely and also indicates that these insertions are stabilized at the population level. Some *Wolbachia* inserts are present at the same position on the chromosomes of both *Cpp* and *Cpe*, while other inserts are subspecies-specific (Fig. 3), indicating that insertion events likely occurred both before and after the divergence of the subspecies in allopatry. *Cpp* and *Cpe*-specific sequences seem to be fairly young since the two subspecies have diverged between 0.2 and 2 MYA (Cooper and Hewitt 1993; Lunt et al. 1998). If future analyses ascertain that hybrid-specific *Wolbachia* inserts originated independently, these would indicate even more recent transfer events given that the hybrid zone appeared approximately 9,000 years ago (Hewitt 1993; Shuker et al. 2005). This scenario seems plausible given that, with an annual generation, these approximately 9,000 generations of hybridization and recombination of parental genomes resulted in a different grasshopper hybrid genome as a consequence of the coadaptation of genomic elements from both subspecies. In the hybrid genomes, new *Wolbachia* transfers may have occurred, while previous insertions could have disappeared or translocated to a different chromosomal position. Further work analyzing individuals from a transect along the hybrid zone will serve to explore these possibilities and will also delineate clines for the new chromosomal markers described here and determine whether they are subject to selection processes.

Previous phylogenetic studies revealed that current *Cp* populations are infected by *Wolbachia* from B and F supergroups and that the F strains co-diverged with their grasshopper hosts in allopatry, as opposed to the more recent horizontal transmission of B strains (Zabal-Aguirre et al. 2010, 2014; Martinez-Rodriguez et al. 2016). This indicates that *Wolbachia* insertions present in the pure grasshopper genomes at the origin of the hybrid zone have further evolved, and perhaps have recombined, as part of the hybrid genome. The current distribution of the *Wolbachia* insertions analyzed here may reflect the history of the insertion events. For example, the Cpar-Wb2 insertion appears in fewer places (8 loci) in *Cpe* than the Cpar-Wb1 or Cpar-Wb3 contigs (10 loci each) but is overrepresented in *Cpp* (12, 8, and 9 loci, respectively, Fig. 3), whilst Cpar-Wb1 and Cpar-Wb3 appear in a similar number of positions in both subspecies (but not always in the same position). These results may indicate that all three inserts were present in the ancestral *Cp* lineage (before the

divergence in allopatry), but we cannot determine if Cpar-Wb2 has been amplified in *Cpp* or partly eliminated in *Cpe*. Further studies are needed to fully understand the evolutionary history of these inserts.

The FISH localizations of the contigs assayed here does not completely correlate with the constitutive heterochromatin distribution described for *Cpp* and *Cpe* (Gosálvez et al. 1988; Bella et al. 1993, 2007; Serrano et al. 1996) (see Fig. 3): pericentromeric heterochromatin present in all the chromosomes of these organisms and the telomeric heterochromatin of chromosome pair 5 in both subspecies never show hybridization. The consistent absence of *Wolbachia* contigs in the pericentromeric areas is interesting and further studies in other grasshoppers and insects are necessary to confirm these observations, especially since we only found one previous reference of pericentromeric *Wolbachia* insertions in the literature (Klasson et al. 2014). Cpar-Wb3 localizes in the previously described telomeric heterochromatin, but it also appears in non-C-heterochromatic positions (in the short arms of L1 and L2 chromosomes in *Cpe* and in interstitial positions in the M4 and M5 chromosomes of both subspecies). However, certain non-C-heterochromatic regions in both chromosome complements show hybridization (see chromosomes L2 and L3, M4, M5 and X in Fig 3). While it seems that the insertions tend to accumulate in the telomeric regions of the chromosomes, there is not a clear pattern of colocalization of the insertions. In general, Cpar-Wb2 and Cpar-Wb3 contigs tend to map together, while all three contigs are localized together in some regions. These positions could be “hot spots” for the insertion of fragments of the *Wolbachia* genome or may indicate a single large insertion of *Wolbachia* DNA (including two or three of these contigs) in the *Cp* genome.

It is noteworthy that the X chromosome in *Cpe* subspecies contains centromeric and interstitial constitutive heterochromatic regions, while in *Cpp* subspecies the constitutive heterochromatin appears in centromeric and telomeric regions. Previous findings using C-banding followed by DNA-specific fluorescent stains proposed that the interstitial heterochromatic band characteristic of the Iberian endemic *Cpe* X chromosome does not derive from an inversion event involving the pericentromeric heterochromatin displayed by this chromosome or the telomeric heterochromatin only present in *Cpp*. They also indicate that the interstitial heterochromatic band specific to the X chromosome of Pyrenean *Cpe* originated recently, perhaps from the amplification and further heterochromatinization of certain unknown sequences (Bella et al. 1993, 2007). The co-localization of this heterochromatin with the three insertions makes these *Wolbachia* sequences possible candidates for the origin of this band. Our current results confirm a non-pericentromeric origin for this interstitial *X-Cpe* heterochromatic band, but it shares all three contigs with the telomeric heterochromatin displayed by the autosomes of both subspecies and Cpar-Wb3 with the telomeric heterochromatin of *X-Cpp* chromosome. The latter region is associated with a NOR only in the *Cpp* subspecies (Gosálvez et al. 1988), and it lacks the Cpar-Wb2 insert commonly present in the non-centromeric ends of the autosomes. However, the same contig appears associated with another NOR region in the short arm of the L2 chromosome of *Cpp* but not in the corresponding region (also with NOR) of *Cpe*, and interstitially appears as an irregular dotted signal in the *X-Cpp* chromosome. This is relevant given that most karyotypic differences in the distribution of *Wolbachia* insertions in these subspecies appear in the X-chromosomes, as has been repeatedly noted for other markers, which

supports the postulated rapid evolution of the sexual chromosomes (van Doorn and Kirkpatrick 2007; Qvarnström and Bailey 2009; Meiklejohn and Tao 2010; Palacios-Giménez et al. 2013) and the preferential accumulation of cytogenetic divergence in these chromosomes during allopatry (Gosálvez et al. 1988; Bella et al. 1993, 2007; Serrano et al. 1996). It is also noteworthy that a reported case of *Wolbachia* lateral transfer to a beetle strictly affects the X chromosome (Kondo et al. 2002; Nikoh et al. 2008), whilst in a tsetse fly, *Glossina morsitans morsitans*, the *Wolbachia* inserts appear distally in the heterochromatic X, Y and B-chromosomes (Brelsfoard et al. 2014), which is similar to what we observe in *Cp*.

With the current data, it is not possible to know if certain C-heterochromatic regions in some way attract repeated *Wolbachia* insertions or if the insertions serve as *foci* for the generation of heterochromatin. Further studies in other organisms would help to ascertain this possible origin for certain heterochromatic regions. This is relevant given the wide distribution of *Wolbachia* in terrestrial arthropods and nematodes (Le Page and Bordenstein 2013) and the ubiquity of lateral transfers from *Wolbachia* to its host.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1. Detection of *Wolbachia* inserts in chromosomes of uninfected individuals of both subspecies of *Cp* (*Cp parallelus*, *Cpp*; *Cp erythropus*, *Cpe*) by tyramide-coupled FISH
 (a, b) Localization of the Cpar-Wb1 *Wolbachia* sequence in *Cpp* chromosomes and *Cpe* chromosomes, respectively. (b, c) Localization of the Cpar-Wb2 *Wolbachia* sequence. (e, f) Localization of the Cpar-Wb3 *Wolbachia* sequence. All the *Wolbachia* sequences are stained in green and chromosomes are counterstained with DAPI (blue). The X chromosome is indicated by an arrow. The signal analyses are performed in diakinesis stage of meiosis. Scale bar 40 μ m.

Fig.2. C-Banding of *Cpp* and *Cpe* chromosomes after detection of Cpar-Wb1 and Cpar-Wb2 *Wolbachia* insert by tyramide-coupled FISH

Green fluorescence reveals localization of Cpar-Wb1 *Wolbachia* insert in the *Cpe* chromosomes (a), and the Cpar-Wb2 insert in the *Cpp* chromosomes (c) counterstained with DAPI. The C bands appear in bright red (stained with propidium iodide), showing a distinctive pattern of constitutive heterochromatic regions for each subspecies, *Cpp* (b) and *Cpe* (d). These different heterochromatic regions allow the proper identification of the X chromosome and discriminate between the L2 and L3 chromosomes (all of them indicated with arrows). Scale bar 40 μ m.

Fig.3. Ideogram of *Cp parallelus* and *Cp erythropus* showing the localization of Cpar-Wb1, Cpar-Wb2, and Cpar-Wb3 inserts

The constitutive heterochromatic regions are represented in pink along the different chromosomes of the two subspecies (Li, L2, L3, M4, M5, M6, S7, S8 and X). The localization of the Cpar-Wb1 insert is denoted in orange, while the Cpar-Wb2 and Cpar-Wb3 inserts are marked in green and red, respectively.