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Characterization of non-cytoplasmic incompatibility inducing *Wolbachia* in two continental African populations of *Drosophila simulans*

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Wolbachia is an endocellular bacterium infecting arthropods and nematodes. In arthropods, it invades host populations through various mechanisms, affecting host reproduction, the most common of which being cytoplasmic incompatibility (CI). CI is an embryonic mortality occurring when infected males mate with uninfected females or females infected by a different *Wolbachia* strain. This phenomenon is observed in *Drosophila simulans*, an intensively studied *Wolbachia* host, harbouring at least five distinct bacterial strains. In this study, we investigate various aspects of the *Wolbachia* infections occurring in two continental African populations of *D. simulans*: CI phenotype, phylogenetic position based on the *wsp* gene and associated mitochondrial haplotype. From the East African population (Tanzania), we show that (i) the *si*III mitochondrial haplotype occurs in continental populations, which was unexpected based on the current views of *D. simulans* biogeography, (ii) the *w*Ki strain (that rescues from CI while being unable to induce it) is very closely related to the CI-inducing strain *w*No, (iii) *w*Ki and *w*No might not derive from a unique infection event, and (iv) *w*Ki is likely to represent the same entity as the previously described *w*Ma variant. In the West African population (Cameroon), the *Wolbachia* infection was found identical to the previously described *w*Au, which does not induce CI. This finding supports the view that *w*Au might be an ancient infection in *D. simulans*.
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Introduction

Wolbachia are maternally transmitted endocellular bacteria infecting arthropods and nematodes (reviewed in Stouthamer *et al*, 1999; Stevens *et al*, 2001). In arthropods, the infection can result in various alterations of sexuality and reproduction such as feminization (Rigaud, 1997), thelytokous parthenogenesis (Stouthamer, 1997), male killing (Hurst *et al*, 1999) and cytoplasmic incompatibility (Hoffmann and Turelli, 1997; Charlat *et al*, 2001). All these phenomena drive infected females to produce more females than uninfected ones, allowing *Wolbachia* to spread and maintain themselves in hosts' populations. The most common phenomenon, cytoplasmic incompatibility (CI), is observed when infected males mate with uninfected females or with females infected by a different, incompatible *Wolbachia* strain. In such crosses, fertilization is apparently normal but subsequent mitoses are disrupted, leading to the death of the zygote (Reed and Werren, 1995; Callaini *et al*, 1996, 1997; Lassy and Karr, 1996). Basically, infected cytoplasms are selected for because the eggs laid by infected females are protected from CI, while those laid by uninfected females are not.

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The mechanism involved is presently unknown, but CI can be interpreted using the *mod*/*resc* model (Werren, 1997), which implies the existence of two bacterial functions: modification (*mod*) and rescue (*resc*). The *mod* function would somehow modify the male pronuclei (Presgraves, 2000), before *Wolbachia* are shed from maturing sperm, and the *resc* function would rescue the embryo through an interaction with modified sperm. An egg fertilized with a modified sperm will not develop normally unless a specific *resc* function is expressed in the egg.

CI is observed in *Drosophila simulans*, an extensively studied *Wolbachia* host (reviewed in Merçot and Charlat, 2003), harbouring several different bacterial variants. Three variants have been shown to induce CI when present in males and to rescue from their own effect when present in females: *w*Ri (Hoffmann *et al*, 1986), *w*Ha (O'Neill and Karr, 1990) and *w*No (Merçot *et al*, 1995). Three other variants have been described that do not seem to induce CI when present in males: *w*Ma (Rousset and Solignac, 1995), *w*Au (Hoffmann *et al*, 1996) and *w*Ki (Merçot and Poinsot, 1998a; Poinsot and Merçot, 1999). Furthermore, *w*Ki has been demonstrated to possess a functional *resc*: eggs infected by *w*Ki are rescued in crosses with *w*No-infected males (Merçot and Poinsot, 1998a; Poinsot and Merçot, 1999). Indirect arguments suggest that *w*Ma would show the same phenotype (Bourtzis *et al*, 1998).

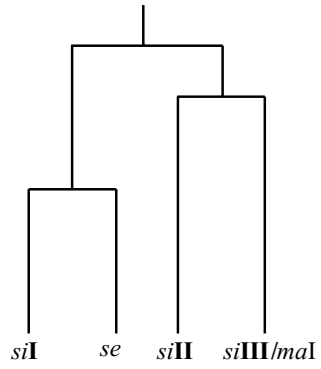

Figure 1 Phylogenetic relationships between mitochondrial haplotypes harboured by *D. simulans* (*si*I, *si*II, *si*III), *D. sechellia* (*se*) and the *ma*I haplotype of *D. mauritiana* (Satta and Takahata, 1990; Ballard, 2000a,b). *se* and *si*I form a monophyletic group, which might be because of the persistence of an ancestral polymorphism in *D. simulans*, or to an introgression event. *ma*I and *si*III are virtually identical, a pattern most likely because of a well-accepted recent introgression (Solignac and Monnerot, 1986; Ballard, 2000c).

These different variants are not randomly associated with the three very distinct mitochondrial haplotypes that have been described in *D. simulans* (*si*I, *si*II and *si*III; Figure 1). This is expected because *Wolbachia* and mitochondria are transmitted together through the egg cytoplasm so that they should remain associated over time (provided that horizontal and/or paternal transmission of *Wolbachia* and/or mitochondria are not too frequent). Thus, the *w*Ri and *w*Au variants are associated with the *si*II haplotype (Hale and Hoffmann, 1990; James and Ballard, 2000), the *w*Ha and *w*No variants are associated with the *si*I haplotype (Montchamp-Moreau *et al*, 1991; Rousset and Solignac, 1995), and the *w*Ma variant is associated with the *si*III haplotype (Rousset *et al*, 1992). As shown here, *w*Ki is also associated with *si*III.

In the present study, two *D. simulans* populations from the African continent were investigated: one from East Africa (Kilimanjaro, Tanzania), known to be infected by *w*Ki (Merçot and Poinsot, 1998a) and one from West Africa (Yaounde, Cameroon). Three different traits were considered: (i) CI phenotype (the *Wolbachia* ability to induce CI or to rescue from it), (ii) sequences of the *Wolbachia Surface Protein* gene and (iii) associated mitochondrial haplotype. Our main conclusions are the following. From the East African population, we show that (i) the *si*III mitochondrial haplotype occurs in continental Africa, which was unexpected based on the current views of *D. simulans* biogeography, (ii) the *w*Ki strain is very closely related to *w*No, (iii) *w*Ki and *w*No might not derive from a unique infection event, and (iv) *w*Ki is likely to represent the same entity as the previously described *w*Ma variant. In the West African population, the *Wolbachia* infection was found to be identical to the previously described *w*Au strain, based on all the traits under study. This finding supports the view that the *w*Au infection in *D. simulans* might be ancient, and raises the question of how non-CI-inducing *Wolbachia* maintain themselves in natural populations.

Materials and methods

Drosophila simulans strains

Reference lines: Agadir is a strain collected in Morocco in 1996, infected by *w*Ri (Poinsot and Merçot, 1999). NHa originates from the Noumea 89 strain, bi-infected by *w*Ha and *w*No. Following segregation of the two variants, NHa only bears *w*Ha (Poinsot and Merçot, 1997). N7No also originates from Noumea 89. Following segregation of the two variants, N7No only bears *w*No (Merçot and Poinsot, 1998b). Coffs Harbour S20 is an Australian strain founded using flies from a 1993 collection, infected by *w*Au (Hoffmann *et al*, 1996). SimO is a naturally uninfected strain from Nasr'allah (Tunisia) (Merçot *et al*, 1995). STC is an inbred stock from the Seychelles strain (Seychelles archipelago), originally bi-infected by *w*Ha + *w*No, cured from its *Wolbachia* following a tetracycline treatment (Poinsot *et al*, 2000). ME29 is a *D. simulans* line transinfected with the *Wolbachia* *w*Mel, naturally infecting the *D. melanogaster* Wien5 isofemale line (Poinsot *et al*, 1998).

Studied lines: Yaounde: 19 isofemale lines have been studied, originating from females collected in Yaounde (Cameroon) in 1997 by B Riera. Kilimanjaro: KC9, K45 and K39 are isofemale lines infected, or originally infected, by *w*Ki. K60 is an isofemale line naturally uninfected. K10P is a mass strain founded using a pool of 10 uninfected isofemale lines. All lines originated from flies collected in 1996 in Tanzania by D Lachaise (Poinsot and Merçot, 1999).

Rearing conditions

During the experiment, all lines were maintained at 25°C in bottles with axenic medium (David, 1962) at low larval competition. For three generations at least before the beginning of CI experiments, all lines concerned were maintained by crossing 20 virgin females aged 4–6 days and 25 virgin males aged 3–4 days in bottles with axenic medium. After 24 h of egg laying, individuals were transferred to a second bottle for another 24 h, before the adults were discarded. Given the laying rates on the strains used, this protocol ensures a low larval competition and (when flies are infected) the maximum expression of CI.

Cytoplasmic incompatibility tests

Individual crosses were carried out using 3-day-old virgin males and 4 to 5-day-old virgin females. Each cross was initiated by placing one male and one female in a vial with axenic medium until mating was observed. The male was then removed and the female was supplied with a laying plate for 48 or 72 h. Upon removal of the female, the eggs were placed at 25°C for 24 h before egg hatch was measured using all eggs. Laying plates containing less than 20 eggs were discarded. All individuals from infected strains were checked by PCR for the presence of *Wolbachia* using 16S primers (O'Neill *et al*, 1992) or general *wsp* primers: 81F and 691R (Braig *et al*, 1998).

wsp sequencing

DNA was extracted from individually crushed flies, using the crude STE boiling method (O'Neill *et al*, 1992).

The *wsp* gene was then amplified by PCR using general primers 81F and 691R (Braig *et al*, 1998). PCR was performed in a 25 µl reaction volume, using 1.25 units of *Taq* DNA polymerase (Perkin Elmer) and 1 µl of DNA template, in the following conditions: 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM reverse and forward primers. Thermal cycles were as follows: 94°C for 1 min, (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) 34 times and 72°C for 5 min. A second PCR was performed in 50 µl reaction volumes with the same concentrations as above, using 2 µl of the first PCR product as template.

The second PCR product was run on a 1% agarose gel. Amplified DNA was then purified using 'Quiaquick Gel Extraction Kit' (Quiagen). Automatic sequencing was done using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems). Each sequence was obtained twice with each primer, making a total of four sequences obtained independently from one DNA extract, from which a consensus was derived. Each base from the final consensus sequence was present in at least three out of the four sequences for every site. Alignment of our sequences with databank sequences was performed using CLUSTALW.

Mitochondrial haplotype determination

Mitochondrial haplotypes (*si*I, *si*II or *si*III) were determined by restriction fragment length polymorphism. DNA was extracted as in Baba-Aïssa *et al* (1988) and digested with restriction enzymes *Hpa* I and *Acc* I, allowing a double-checking of the haplotypes. *si*I, *si*II and *si*III were distinguished using restriction maps from Baba-Aïssa *et al* (1988).

Results

Prevalence and CI assays

The prevalence and CI phenotype in the East African population (Kilimanjaro, Tanzania) are known from a previous study (Merçot and Poinsot, 1998a): 9 lines out of 49 were found infected (prevalence 18.4%) and the *Wolbachia* variant present in this population, baptized *w*Ki, does not induce CI but is capable of rescuing the CI induced by *w*No.

Isofemale lines from the West African population (Yaounde, Cameroon) were screened by PCR for the presence of *Wolbachia*. Six out of 19 were found to be infected (prevalence: 32%). The CI assays described below were performed using these infected lines.

In order to detect the possible expression of CI in Yaounde lines (*mod* test), males from five infected and five uninfected isofemale lines were individually crossed with two types of uninfected females: from an uninfected reference strain (SimO) and from a pool of Yaounde uninfected lines (massY−). The results, presented in Figure 2a, were analysed using a nonparametrical Wilcoxon test. As presented in Table 1A, no expression of CI was detected: with both SimO and mass Y− females, infected males are not significantly less fertile than uninfected ones.

In order to determine if the *Wolbachia* present in Yaounde was able to rescue the CI induced by other strains (*resc* test), females from five infected and five uninfected isofemale lines from Yaounde were crossed with males infected by each of the three CI-inducing *Wolbachia* variants naturally infecting *D. simulans* (*w*Ri, *w*Ha and *w*No) and with males from the ME29 line (a *D. simulans* line transinfected with *w*Mel, the *Wolbachia* naturally infecting *D. melanogaster*; Poinsot *et al*, 1998). The results, shown in Figure 2b, were analysed using a Wilcoxon test. As presented in Table 1B, no rescue was detected: with all types of males (infected by *w*Ri, *w*Ha, *w*No or wMel), infected females are not significantly more fertile than uninfected ones.

We finally tested whether the presence of *Wolbachia* in the Yaounde population affected female fertility. Females from the massY− (uninfected) and massY+ (infected) were crossed with uninfected and infected males. The results, presented in Figure 2c, were analysed using a Wilcoxon test. As shown in Table 1C, no effect on fertility was detected: with both infected and uninfected males, infected females are not significantly more or less fertile than uninfected ones.

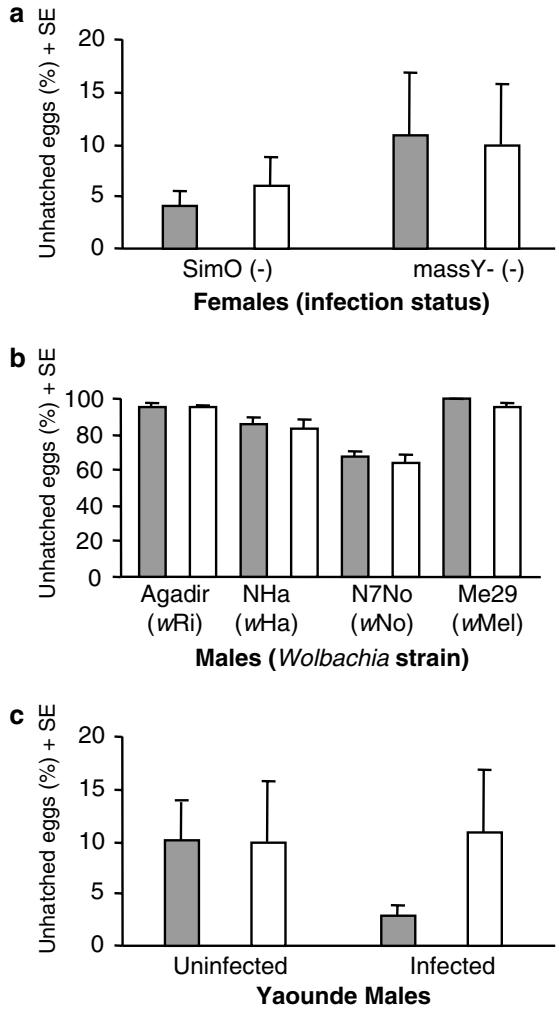

Figure 2 Results of crosses realized with the Yaounde population. A total of 15 replicates were obtained for each category of cross. (a) *mod* test, involving Yaounde males, infected (grey) and uninfected (white); (b) *resc* test, involving Yaounde females, infected (grey) and uninfected (white); (c) fertility test, involving Yaounde females, infected (grey) and uninfected (white).

Table 1 Results of Wilcoxon tests

	Question addressed	*Comparison*[a]			*W*	*P*
		Male × Fem	*versus*	*Male × Fem*		
A	Mod?	Y+ × SimO	vs	Y− × SimO	0.249	<0.81
		Y+ × massY−	vs	Y− × massY−	0.124	<0.91
B	Resc?	Agadir × massY−	vs	Agadir × massY+	0.933	<0.36
		NHa × massY−	vs	NHa × massY+	0.601	<0.55
		N7No × massY−	vs	N7No × massY+	0.27	<0.79
		Me29 × massY−	vs	Me29 × massY+	1.327	<0.19
C	Effect on female fertility?	Y− × massY−	vs	Y− × massY+	0.85	<0.4
		Y+ × mass Y−	vs	Y+ × mass Y+	1.203	<0.24

[a]Y+ and Y− are males from the Yaounde population, infected and uninfected, respectively. Fem: female. W: result of the Wilcoxon test.

wsp sequences

wsp gene sequences were determined from two West African lines (Y6 and Y12), one East African line (KC9, infected by *w*Ki), as well as in ME29 (infected by *w*Mel) and Coffs Harbour S20 (infected by *w*Au).

The sequence length was 598 bp for Y6, Y12, Coffs Harbour S20 and ME29. The Coffs Harbour S20 sequence was, as expected, identical to the one obtained by Zhou *et al* (1998) using the same line (GenBank AF020067). The Y6 and Y12 sequences (GenBank AF290890) were identical to the Coffs Harbour S20 sequence. The ME29 sequence (GenBank AF290891) was identical to some of the previously determined *wsp* sequences obtained from *D. melanogaster* by Zhou *et al* (1998) (GenBank AF020063, AF020064, AF020065, AF020072). The Y6, Y12 and Coffs Harbour S20 sequences differed by five substitutions from the ME29 sequence.

The sequence length was 566 bp for KC9. The KC9 sequence (GenBank AF290889) was identical to the *w*No and *w*Mau sequences previously obtained by Zhou *et al* (1998) (GenBank AF020074 and AF020069). Let us note here that the AF020069 sequence (Zhou *et al*, 1998) was obtained using a *D. simulans* line artificially infected by *w*Mau (Giordano *et al*, 1995), the *Wolbachia* strain naturally infecting *D. mauritiana*. Since *w*Mau and *w*Ma are closely related (Rousset and Solignac, 1995), Zhou *et al* (1998) term this strain *w*Ma.

Mitochondrial haplotypes

Mitochondrial haplotypes were determined in four West African isofemale lines (Y6 and Y12, infected; Y4 and Y5, uninfected), in four East African isofemale lines (K45, KC9, K39, originally infected; K60, originally uninfected), in one East African mass strain (K10P, originally uninfected), as well as in Coffs Harbour S20 and SimO (*si*II references) and STC (*si*I reference).

As expected from previous typing (Montchamp-Moreau *et al*, 1991; James and Ballard, 2000), we found that SimO, Coffs Harbour S20 and STC harboured, respectively, *si*II, *si*II and *si*I. West African lines harboured *si*II, regardless of their original infection status. East African isofemale lines harboured *si*III, regardless of their original infection status. The East African mass strain, originally uninfected, was heterogeneous, harbouring *si*III as well as *si*II cytoplasms.

Discussion

*si*III mitochondrial haplotype occurs in continental populations

The three distinct mitochondrial haplotypes of *D. simulans* show a very strong geographic structuration, on the basis of which biogeographical inferences have been made (Lachaise *et al*, 1988). The classical view is that (i) *si*I is restricted to the Seychelles archipelago and Indo-Pacific islands, (ii) *si*II is much more widely distributed, occurring in all continental populations, as well as in Madagascar and La Reunion islands, and (iii) *si*III is restricted to Madagascar and La Reunion islands (Solignac and Monnerot, 1986; Baba-Aïssa *et al*, 1988; Montchamp-Moreau *et al*, 1991; Ballard, 2000b).

We have determined the mitochondrial haplotype of several lines from the Kilimanjaro population (Tanzania). The four isofemale lines were found to harbour the *si*III haplotype, while a pool from the same area was found to be polymorphic, with *si*II and *si*III cytoplasms. This is not the first report that the *si*II and *si*III cytoplasms can be found in sympatry: this situation occurs in Madagascar and La Reunion (Baba-Aïssa *et al*, 1988; Ballard, 2000b). However, the *si*III haplotype had never been observed in continental populations. This finding suggests that, at least at the mitochondrial level, continental and insular populations are not differentiated. Consistent with our finding are some recent results based on the *vermillion* locus suggesting that continental and island populations from East Africa are also similar at the nuclear level (N. Derome, personal communication). A more systematic screening of mitochondrial haplotypes in continental East African populations could show whether the pattern we report here reflects a very general or only localized situation. Let us finally mention here that in an earlier paper (Nigro, 1994), the SimO strain (Nasr'allah, Tunisia) was mistakenly reported to harbour the *si*III mitochondrial variant (instead of *si*II), owing to an unfortunate confusion between strain names.

*w*Ki and *w*No are closely related, but might not derive from a unique infection event

The [*mod*− *resc*+] phenotype, where *Wolbachia* does not induce CI but is capable of rescuing it, was initially described in the Kilimanjaro population (Merçot and

Poinsot, 1998a; Poinsot and Merçot, 1999) using the lines investigated in the present study. The *Wolbachia* strain responsible for this phenotype was baptized *w*Ki. In these studies, it was shown that *w*Ki, when present in males, does not induce CI, but, when present in females, rescues the embryonic mortality induced by *w*No, a relationship that was confirmed in additional experiments (Charlat *et al*, 2002). The authors thus characterized *w*Ki through CI assays, the results of which suggested that *w*Ki and *w*No might be closely related. However, neither the precise phylogenetic position of *w*Ki nor the mitochondrial haplotype associated with this infection were determined. Our sequence results show that *w*Ki and *w*No bear the same *wsp* sequence. Thus, as expected from their CI relationships, and provided that recombination is not misleading us (Werren and Bartos, 2001; Jiggins *et al*, 2001; Charlat and Merçot, 2001), these two *Wolbachia* strains are very closely related.

We found that all the infected (or originally infected) lines from Kilimanjaro harbour the *si*III mitochondrial haplotype. As mentioned above, such a result was unexpected based on the geographical origin of this strain. It was also unexpected on the basis of the molecular resemblance between *w*No and *w*Ki: since *w*No is associated with the *si*I mitochondrial haplotype, a reasonable prediction was that the same would be true for *w*Ki. The hypothesis that *w*Ki and *w*No could derive from a unique infection event, having occurred within the *si*I lineage, is ruled out.

Based on our results, could *w*No and *w*Ki result from a divergence associated with the *si*I/*si*III split? In other words, could these two bacterial variants derive from a unique and ancestral infection event, having occurred prior to the coalescence between the three mitochondrial haplotypes? Under such a view, the *w*No/*w*Ki strain would have been subsequently lost from the *si*II lineage, since the *si*II and *si*III haplotypes form together a monophyletic group (Figure 1). We think this scenario is somewhat unlikely. Indeed, *w*No and *w*Ki are very closely related: identical *wsp* sequences and one substitution over 800 bp on the 16S rRNA sequence (A James and J Ballard, personal communication). By contrast, the *si*I and *si*III haplotype are significantly divergent: 355 nucleotide substitutions over 14 959 bp (2.4% divergence) (Ballard, 2000a). In *Drosophila*, mitochondria seem to evolve at a faster rate than nuclear genes (Moriyama and Powell, 1997), but it has also been suggested that endocellular bacteria have increased substitution rates (Clark *et al*, 1999). The discrepancy between *Wolbachia* and mitochondrial divergence would thus make more likely the hypothesis of a recent horizontal transfer. This interpretation must however be considered cautiously, as it does not rely on well-calibrated molecular clocks.

Theory suggests that nothing opposes the decrease of CI intensity within a population of CI-inducing *Wolbachia*. Indeed, although CI allows *Wolbachia* to invade host populations, any mutant clone inducing a lower CI, or no CI at all, would not be selected against, as long as the *resc* function is maintained (Prout, 1994; Turelli, 1994; Hurst and McVean, 1996). Accordingly, it has been suggested that non-CI-inducing *Wolbachia* could derive from CI-inducing ones. The fact that *w*No and *w*Ki are so closely related suggests that a shift between the [*mod*+] and [*mod*−] phenotypes can occur within a relatively brief period of time.

*w*Ki and *w*Ma represent the same entity

The *w*Ma *Wolbachia* strain was initially described from Madagascar (Rousset *et al*, 1992) as a non-CI-inducing strain (Rousset and Solignac, 1995). Based on 16S rRNA sequences, a slowly evolving marker, these authors showed that *w*Ma and *w*No are closely related. However, the CI relationships between *w*Ma and *w*No were not investigated. In fact, lines singly infected by *w*No were not available until this variant was isolated by segregation from doubly infected lines (Merçot *et al*, 1995).

Let us consider the following list of arguments, strongly suggesting that *w*Ki and *w*Ma represent the same entity. (i) *w*Ma and *w*Ki show identical *wsp* sequences (Zhou *et al*, 1998; this study), as well as identical 16S sequences (Rousset *et al*, 1992; A James and J Ballard, personal communication). (ii) *w*Ma and *w*Ki are both associated with the *si*III mitochondrial haplotype (Rousset and Solignac, 1995; James and Ballard, 2000; this study). (iii) *w*Ma and *w*Ki are both non-CI-inducing strains (Rousset and Solignac, 1995; Merçot and Poinsot, 1998a). (iv) On the basis of mitochondrial haplotypes, it is well accepted that a recent introgression took place between *D. simulans* and *D. mauritiana* (Solignac and Monnerot, 1986; Ballard, 2000c), allowing the *si*III haplotype, together with the *w*Ma *Wolbachia* strain, to invade *D. mauritiana*. Accordingly, the *Wolbachia* strain occurring in *D. mauritiana*, usually referred to as *w*Mau, is identical to *w*Ma, on the basis of the 16S rRNA (Rousset and Solignac, 1995). Just as *w*Ma, *w*Mau does not induce CI (Giordano *et al*, 1995; Rousset and Solignac, 1995). However, it appears that *w*Mau, when injected into *D. simulans*, is able to rescue the CI induced by *w*No (Bourtzis *et al*, 1998). Thus, *w*Mau and *w*Ki show the same CI phenotype, indirectly suggesting that the same could be true for *w*Ma and *w*Ki. Based on these different arguments, we believe that *w*Ki and *w*Ma represent the same entity. Since the *w*Ma name was published first, we recommend referring to *w*Ki as '*w*Ma' in future publications.

*w*Au is in West Africa

The *w*Au infection was originally reported in Australia (Hoffmann *et al*, 1996) and more recently in Madagascar and Florida, USA (James and Ballard, 2000). Its presence is also suspected, although not clearly demonstrated, in Ecuador (Turelli and Hoffmann, 1995). Based on this geographical distribution, the *si*II mitochondrial haplotype was expected, and indeed observed by James and Ballard (2000). We found the *si*II haplotype in the Coffs Harbour S20 line, confirming this result.

The *Wolbachia* strain that we found in populations from Cameroon seems identical to *w*Au: (i) the two strains harbour the same *wsp* sequence, (ii) they are both associated with the *si*II haplotype and (iii) they do not induce CI (Hoffmann *et al*, 1996), nor are they able to rescue CI from any of the CI-inducing *Wolbachia* naturally infecting *D. simulans* (Merçot and Poinsot, 1998b), or *w*Mel, injected from *D. melanogaster* into *D. simulans* (Poinsot *et al*, 1998).

D. simulans non-African populations are thought to result from a recent expansion of the species (Lachaise *et al*, 1988). In other words, the Yaounde population, where we observed *w*Au, is probably older than Australian or American populations that have pre-

viously been found infected by this variant. Supporting this view are some results based on the *vermillion* nuclear gene, confirming that flies from the Yaounde population are probably not reintroduced from recently colonized areas (Hamblin and Veuille, 1999). The presence of *w*Au, a non-CI-inducing strain, in ancient populations – Cameroon (this study), but also Madagascar (James and Ballard, 2000) – is consistent with current views on CI evolution. Indeed, since the [*mod*−] phenotype is expected to derive from the [*mod*+] phenotype (Prout, 1994; Turelli, 1994; Hurst and McVean, 1996), [*mod*−] strains should, in general, be more ancient than [*mod*+] ones. If, as we suspect, *w*Au has been present for a long time in *D. simulans*, this infection should be associated with a high diversity of mitochondrial haplotypes, unless recent selective sweeps occurred. A study including *w*Au-infected flies from Madagascar does not support this prediction: mitochondrial genomes associated with *w*Au cluster together in a narrow monophyletic group (Ballard, 2000b). Including flies from Australia, America and West Africa in such an analysis might clarify this issue.

Non-CI-inducing *Wolbachia* are widespread in *D. simulans*

The role played by CI in the spread of *Wolbachia* has been extensively modelled (reviewed in Hoffmann and Turelli, 1997) and witnessed in real time in the wild (Turelli and Hoffmann, 1995). However, it appears that non-CI-inducing strains can also be maintained in natural populations, as suggested by the widespread occurrence of *w*Au and *w*Ki/*w*Ma. This apparently paradoxical situation might not be so if the transmission from mothers to offspring is perfect, as observed in Australian populations, in which case *Wolbachia* infection would simply behave as a neutral variant (Hoffmann *et al*, 1996). In West African lines, however, it seems that *w*Au is not perfectly transmitted: uninfected individuals are often collected from initially infected isofemale lines (unpublished results), and the same is true from *w*Ki/*w*Ma. If, as we suspect, transmission is not perfect, other factors, such as positive effects on host fitness, high rates of horizontal transmission, or other reproductive phenotypes, might have to be hypothesized and tested.

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