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Molecular data reveal a cryptic species within the *Culex pipiens* mosquito complex

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Abstract

The *Culex pipiens* mosquito complex is a group of evolutionarily closely related species including *C. pipiens* and *Culex quinquefasciatus*, both infected by the cytoplasmically inherited *Wolbachia* symbiont. A *Wolbachia*-uninfected population of *C. pipiens* was however described in South Africa and was recently proposed to represent a cryptic species. In this study, we reconsidered the existence of this species by undertaking an extensive screening for the presence of *Wolbachia*-uninfected *C. pipiens* specimens and by characterizing their genetic relatedness with known members of the complex. We first report on the presence of *Wolbachia*-uninfected specimens in several breeding sites. We next confirm that these uninfected specimens unambiguously belong to the *C. pipiens* complex. Remarkably, all uninfected specimens harbour mitochondrial haplotypes that are either novel or identical to those previously found in South Africa. In all cases, these mitochondrial haplotypes are closely related, but different, to those found in other *C. pipiens* complex members known to be infected by *Wolbachia*. Altogether, these results corroborate the presence of a widespread cryptic species within the *C. pipiens* species complex. The potential role of this cryptic *C. pipiens* species in the transmission of pathogens remains however to be determined. The designation '*Culex juppi* nov. sp.' is proposed for this mosquito species.

Keywords: *Wolbachia*, *Culex pipiens* mosquito complex, cytoplasmic incompatibility, mitochondria.

Introduction

Cytoplasmically inherited symbionts are widespread in arthropods (Werren & Windsor, 2000; Weinert *et al.*, 2007; Duron *et al.*, 2008a,b). They are typically transmitted only by female hosts through the egg cytoplasm, males being a dead end in term of transmission (Moran *et al.*, 2008; Werren *et al.*, 2008). The most common of them, the alpha-proteobacterium *Wolbachia*, is usually termed a 'reproductive parasite' in the sense that it optimizes its transmission by manipulating the host's reproductive biology (Werren *et al.*, 2008; Engelstädter & Hurst, 2009; Cordaux *et al.*, 2011). In many host species, *Wolbachia* has evolved a conditional sterility phenotype, known as cytoplasmic incompatibility (CI) (Werren *et al.*, 2008; Engelstädter & Telschow, 2009). In its simplest form, it specifically kills the embryos of uninfected females mated with infected males, whereas the other direction of the cross (infected females mated with uninfected males) produces viable progeny, ie unidirectional CI. This phenomenon provides a reproductive advantage to infected females and favours the spread of *Wolbachia* in host populations. In more complex cases, CI can also occur between males and females carrying incompatible *Wolbachia* strains, with crossing relationships exhibiting either unidirectional CI or bidirectional CI (both directions of a cross are sterile). Aside from CI, the spread of *Wolbachia* is also influenced by antagonist forces, such as an infection cost imposed on female hosts and imperfect transmission of *Wolbachia* to the eggs (Hoffmann *et al.*, 1990; Engelstädter & Telschow, 2009). Taken together, these parameters determine an invasion threshold for CI; that is, an infection frequency below which *Wolbachia* becomes extinct and above

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which it invades, typically until fixation (Hoffmann *et al*., 1990; Engelstädter & Telschow, 2009).

Mosquitoes of the *Culex pipiens* complex are naturally infected by a variety of CI-inducing *Wolbachia* strains belonging to the *w*Pip clade (Rasgon & Scott, 2003; Duron *et al*., 2006b; Atyame *et al*., 2011a). This system is characterized by a rapid diversification of CI determinants (Duron *et al*., 2012; Nor *et al*., 2013) that has led to an unrivalled variety of crossing types, including uni- and bi-directionally incompatible *w*Pip strain types (Guillemaud *et al*., 1997; Duron *et al*., 2006a; Atyame *et al*., 2011b; Atyame *et al*., 2014). The two most widespread members of this species complex are the common house mosquito, *C. pipiens*, and the southern house mosquito, *Culex quinquefasciatus* (Vinogradova, 2000; Smith & Fonseca, 2004; Farajollahi *et al*., 2011). The first one, *C. pipiens*, is common in temperate regions and is subdivided into two subspecies, *Culex pipiens pipiens* (Europe and North and South Africa) and *Culex pipiens pallens* (East Asia). In addition, two recognized forms, '*pipiens*' and '*molestus*', are also encountered in *C. p. pipiens* in the Northern Hemisphere. The second species, *C. quinquefasciatus*, is rather found across the tropics and the lower latitudes of temperate regions. Both species, including all the subspecies and forms, are infected by *w*Pip with infection frequencies near or at fixation in field populations (Rasgon & Scott, 2003; Duron *et al*., 2005; Dumas *et al*., 2013). This infection pattern is well explained by the ability of *w*Pip-infected males to induce complete CI with uninfected females, a near perfect maternal transmission of infection and a reduced effect on female fecundity (Rasgon & Scott, 2003; Duron *et al*., 2006c). Two other species are currently recognized within this complex, but they remain poorly studied: *Culex australicus* and *Culex globocoxitus*, which are both restricted to Australia (Smith & Fonseca, 2004; Farajollahi *et al*., 2011) and are not infected by *Wolbachia* (Irving-Bell, 1974).

The pattern of mtDNA variation within the *C. pipiens* complex is known to be confounded by the spread of *Wolbachia*: both are linked through maternal cotransmission within egg cytoplasm, resulting in complete linkage disequilibrium of mtDNA with *w*Pip infection (Rasgon *et al*., 2006; Atyame *et al*., 2011a; Dumas *et al*., 2013). The invasion of the *w*Pip ancestor within the last 20 000 years resulted in an indirect selective sweep of the mtDNA, which has led to the loss of mtDNA variation within host populations and erased any geographical structure (Rasgon *et al*., 2006; Atyame *et al*., 2011a; Dumas *et al*., 2013). Furthermore, occasional hybridization events resulted in cytoplasmic introgression of both *w*Pip and associated mtDNA between *C. pipiens* and *C. quinquefasciatus* populations, and ultimately led to the global homogenization of mtDNA variation between the two species (Atyame *et al*., 2011a; Dumas *et al*., 2013). Therefore, although each species has a unique genetic signature at nuclear loci (Fonseca *et al*., 2004; Smith & Fonseca, 2004), they cannot be distinguished on the basis of their mtDNA as the pattern of mtDNA variation reflects the evolutionary history of *w*Pip infection rather than of the mosquito populations (Rasgon *et al*., 2006; Atyame *et al*., 2011a; Dumas *et al*., 2013).

Forty years ago, Irving-Bell (1977) reported the absence of *Wolbachia* in southern African *C. pipiens* (SAP) specimens based on microscopic observations. More recently, Cornel *et al*. (2003) also described a *Wolbachia*-uninfected SAP population, indicating that the absence of *Wolbachia* infection was persisting in this region. Remarkably, the SAP specimens were found to be morphologically indistinguishable from the *Wolbachia*-infected *C. pipiens* found in the Northern Hemisphere, and reproductively isolated from sympatric *C. quinquefasciatus* infected populations (Jupp, 1978; Cornel *et al*., 2003). Rasgon *et al*. (2006) further characterized higher mtDNA haplotype diversity in the SAP population relative to other populations of the *C. pipiens* complex. It was thus hypothesized that the uninfected SAP population may represent a cryptic species within the *C. pipiens* complex, within which *Wolbachia* introgression has been prevented by reproductive isolation, maintaining ancestral levels of mtDNA diversity (Rasgon *et al*., 2006). To date, the SAP population is however the single geographical record of this cryptic species.

Here, we reconsidered the existence of cryptic species in the *C. pipiens* complex by (1) undertaking an extensive screening for the presence of *Wolbachia*-uninfected *C. pipiens* specimens; (2) characterizing nuclear and mtDNA lineages of uninfected specimens through a multilocus typing scheme; and (3) estimating their relatedness with known members of the complex, including the uninfected SAP population. Using this approach, we thus attempted to infer the evolutionary processes shaping the species diversity within this mosquito complex.

Results

Distribution of uninfected specimens

Three hundred and forty eight specimens from eight breeding sites located in Europe (two sites in Scotland, UK, and one in Corsica, France) and North Africa (five sites in Tunisia) were screened for the presence of *Wolbachia* (Table 1). Of the 348 specimens, 163 (47%) were found to be uninfected using the *Wolbachia surface protein (wsp)* and the *Ankyrin domain protein (ank2)* PCR assays. The presence of both infected and uninfected specimens was further confirmed by real-time quantitative PCR (qPCR) assays: *Wolbachia* was then detected in four specimens previously diagnosed as positive (on the basis

Table 1. List of mosquito breeding sites examined in this study

Breeding sites	N	Frequency of *Wolbachia*-uninfected specimens (N uninfected)
Quest (Scotland, 2002)	8	0.25 (2)*
Field (Scotland, 2012)	92	0.01 (1)*
Corsica (France, 1993)	18	0.11 (2)*
Kef (Tunisia, 2008)	30	0.43 (13)*
Boussalem (Tunisia, 2008)	42	0.48 (20)*†
Mateur (Tunisia, 2008)	50	0.58 (29)*†
Souala (Tunisia, 2008)	60	0.92 (55)*
Zerga (Tunisia, 2010)	48	0.92 (41)*

N, number of specimens.
**Wolbachia* infection status was diagnosed using both Wolbachia surface protein (*wsp*) and the Ankyrin domain protein (*ank2*) PCR assays.
†*Wolbachia* infection status was diagnosed in a subsample of specimens using real-time quantitative PCR assays.

of *wsp* and *ank2* PCR assays) but not in 11 other specimens previously diagnosed as negative. Overall, uninfected specimens were thus detected in each examined site with a frequency ranging from rare (0.01) to common (0.92; Table 1). Frequency of uninfected specimens was not homogeneous amongst breeding sites as significant variation occurs amongst them (Fisher's exact test, $P = 2\times10^{-16}$): uninfected specimens were more common in Tunisian breeding sites (158 uninfected specimens of 230 examined) than in European sites (five of 118; Fisher's exact test, $P = 2\times10^{-19}$).

Evolutionary origin of uninfected specimens

We further examined the evolutionary relationships of European and North African uninfected specimens with other members of the *C. pipiens* complex, including SAP, and with other *Culex* species (Table S1). We included in the phylogenetic analyses nuclear internal transcribed spacer 2 (ITS2) and acetylcholinesterase-2 (*ace2*) sequences (351 and 529 bp unambiguously aligned nucleotide sites, respectively) and mitochondrial nicotinamide adenine dinucleotide (*NADH*) *dehydrogenase subunit 2* (*ND2*; 329 bp), *ND4* (287 bp) and *cytochrome oxidase I* (*COI*; 450 bp) sequences. In total, 98 new sequences from nuclear and mitochondrial markers have been deposited in GenBank. We identified from our uninfected specimens three ITS2 haplotypes, one *ace-2* haplotype, five *ND2* haplotypes, three *ND4* haplotypes and six *COI* haplotypes. When the sequences were examined separately for each gene, maximum likelihood (ML) analyses were all globally congruent with the current *Culex* classification: we recovered the clustering of *C. p. pipiens*, *C. p. pallens* and *C. quinquefasciatus* within the *C. pipiens* complex and the presence of *Culex torrentium* as the closest relative of the complex whereas other *Culex* species are more distantly related (Figs 1–3, S1–S3), in agreement with previous phylogenetic investigations (Miller *et al*., 1996; Severini *et al*., 1996).

All the European, Tunisian and SAP uninfected specimens proved to be phylogenetically closely related to the known *C. pipiens* members and all clearly fall within the complex as further detailed below. On the basis of ITS2 ML analysis, all *C. pipiens* complex members cluster with uninfected specimens, a pattern highlighting their common evolutionary origin (Fig. 1). The ITS2 sequences however exhibit insufficient polymorphism between infected and uninfected specimens, preventing characterization of a clear genetic structure within the *C. pipiens* complex. By contrast, the *ace-2* sequences are more polymorphic between *C. pipiens* complex members and the ML analysis is thus more discriminative. It clearly separates *C. p. pipiens* from *C. quinquefasciatus* and from *C. australicus* (Fig. 2), as also observed in previous studies (Bourguet *et al*., 1998; Smith & Fonseca, 2004). Worthy of note is that the ML analysis recovered the clustering of the *C. quinquefasciatus* and *C. p. pallens ace-2* sequences; this was at first sight surprising, but is actually expected as hybridization occurs between these two taxa and the *C. quinquefasciatus ace-2* alleles are known to have widely introgressed within the *C. p. pallens* populations (Fonseca *et al*., 2009), resulting in the pattern observed in Fig. 2. Remarkably, on the basis of *ace-2* sequences, all uninfected specimens cluster with *C. p. pipiens* specimens (which are *Wolbachia*-infected) and are thus more closely related to this subspecies than to any other members of the *C. pipiens* complex. None of the uninfected specimens we examined here is closely related to *C. australicus* (Fig. 2), which is known to be not infected by *Wolbachia* (Irving-Bell, 1974).

Examination of each of the mtDNA markers (Figs S1–S3), as well as the *ND4*, *ND2* and *COI* concatenated set (Fig. 3), unambiguously discriminates uninfected specimens from other *C. pipiens* complex members. The same *ND4* haplotype was observed amongst all of the *Wolbachia*-infected *C. pipiens* members: *C. p. pipiens*, *C. p. pallens* and *C. quinquefasciatus* (Fig. S1). By contrast, three distinct *ND4* haplotypes (93.0 to 97.9% pairwise identity; differing by six to 20 positions over 287 bp) were found in the European and

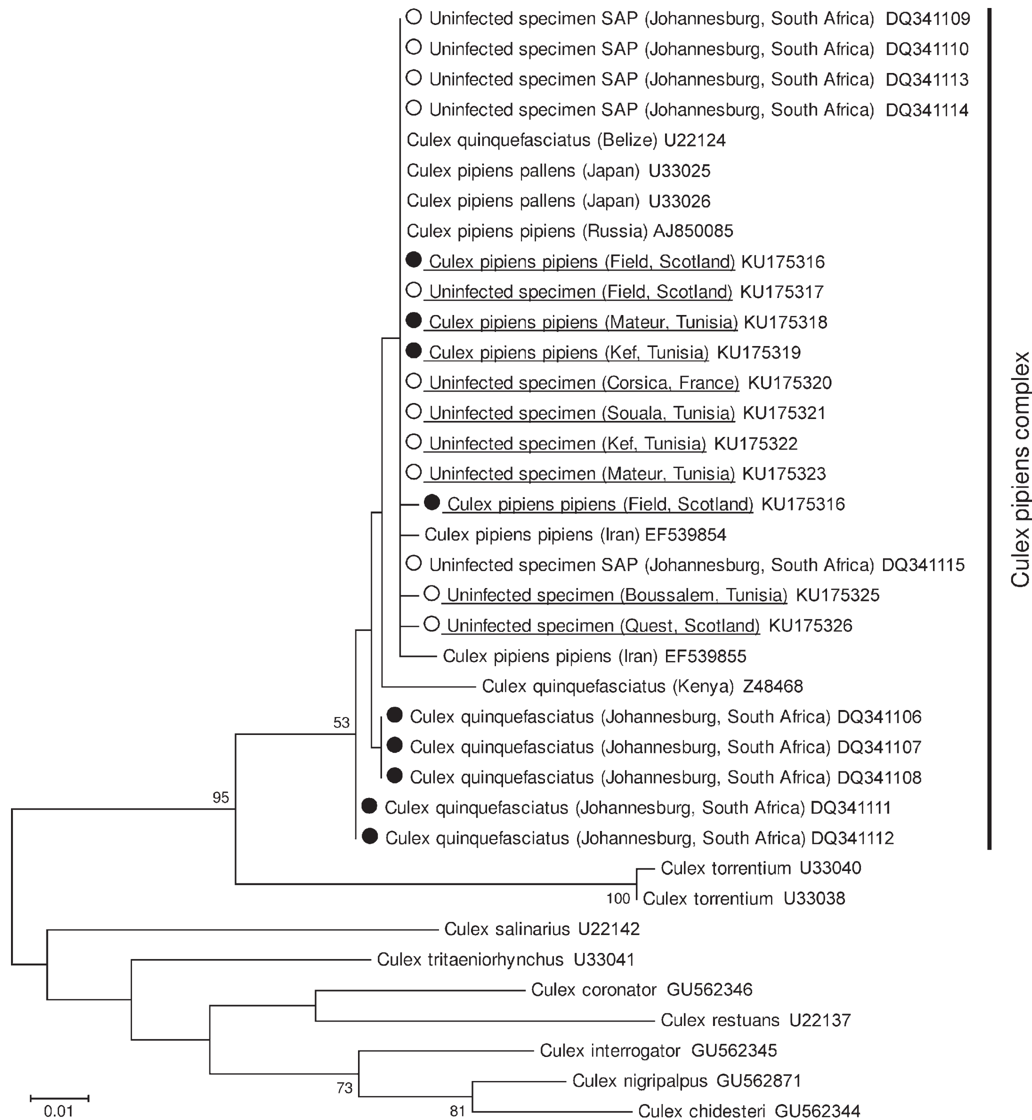

Figure 1. Mosquito internal transcribed spacer 2 phylogenetic tree constructed using the maximum parsimony method. Sequences from this study are underlined; other sequences are from GenBank (listed in Table S1). White circles: uninfected specimens from Scotland, France, Tunisia (this study) and South Africa (Rasgon *et al.*, 2006). Black circles: *Wolbachia*-infected specimens from Scotland, France, Tunisia (this study) and South Africa (Rasgon *et al.*, 2006). Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 50 are shown. The scale bar is in units of substitution/site. GenBank numbers are specified for each sample.

North African uninfected specimens and none showed complete identity to the one present in *Wolbachia*-infected specimens (91.5 to 98.6% pairwise identity between uninfected and *Wolbachia*-infected specimens; differing by four to 24 positions). Remarkably, one of the *ND4* haplotypes found in uninfected North African specimens shows complete identity with one SAP *ND4* haplotype (Fig. S1). Similarly, *ND2* and *COI* sequences were also much more variable amongst uninfected specimens than amongst infected members of the *C. pipiens* complex (Figs S2, S3) although a comparison with SAP specimens was not possible (no SAP *ND2* and *COI* sequences are available from previous studies as only SAP *ND4* have been sequenced; cf. Rasgon *et al.*, 2006). The analysis of *ND4*, *ND2* and *COI* concatenated sequences (1080 bp unambiguously aligned nucleotide sites) revealed a total of seven mtDNA multilocus haplotypes (95.8–98.9% pairwise identity) specific to uninfected specimens (Fig. 3). Furthermore, mitochondrial haplotype relationships analysis confirmed an unambiguous differentiation between uninfected specimens and other *C. pipiens* complex members (Fig. S4).

As occasional hybridization events have resulted in a *w*Pip-driven cytoplasmic introgression of associated mtDNA between *C. pipiens* and *C. quinquefasciatus* populations, these two species cannot be distinguished

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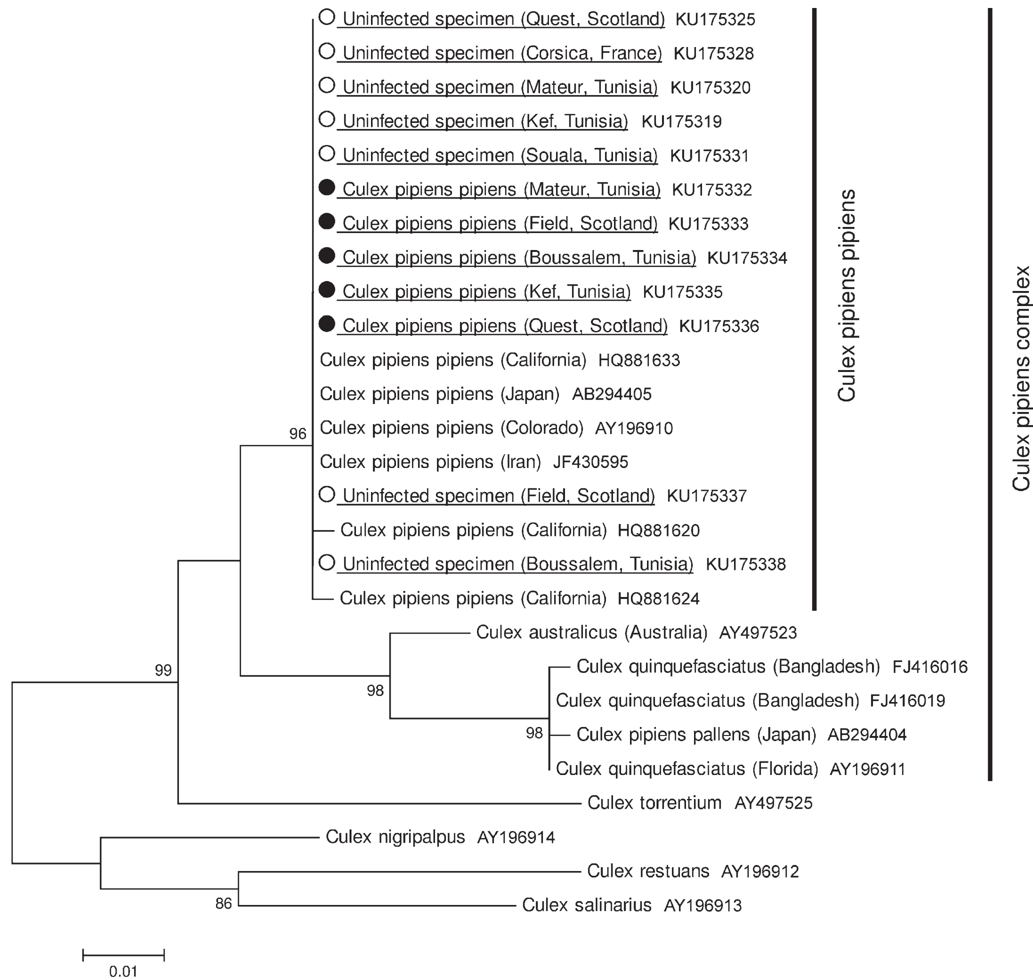

Figure 2. Mosquito acetylcholinesterase-2 (*ace2*) phylogenetic tree constructed using the maximum parsimony method. Sequences from this study are underlined; other sequences are from GenBank (listed in Table S1). White and black circles represent uninfected and *Wolbachia*-infected specimens from this study, respectively. Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown. GenBank numbers are specified for each sample.

on the basis of their mtDNA sequences (Atyame *et al.*, 2011a; Dumas *et al.*, 2013). As a result of this global cytoplasmic homogenization, all of the mtDNA sequences of infected mosquitoes cluster in a monophyletic subclade (Figs 3, S1–S3). At least four main mtDNA subclades (labelled A to D hereafter) can be distinguished within the *C. pipiens* complex and it is obvious that this mtDNA structure mirrors the *Wolbachia* infection status: whereas the A subclade encompasses all mtDNA sequences of infected mosquitoes, the B, C and D subclades only contain mtDNA sequences of uninfected mosquitoes (Fig. 3). The mtDNA diversity of the B, C and D subclades fits at least partially with the geographical origins of uninfected specimens: the B subclades was found in France and Tunisia, C only in Scotland and D only in Tunisia. Worthy of note is that, on the basis of the *ND4* phylogeny, all of the SAP specimens are more closely related to the uninfected specimens from Tunisia belonging to the D subclade than to those of the other subclades (Fig. S1).

Discussion

Our results illustrate the complexity of taxonomic relationships amongst members of the *C. pipiens* complex, and show that differences in *Wolbachia* infection status between sympatric specimens are important indicators of population structure. We observed the presence of *Wolbachia*-uninfected *C. pipiens* specimens in several breeding sites in Europe and North Africa. Using a multilocus typing scheme, we further confirmed that these uninfected specimens unambiguously belong to the *C. pipiens* complex and on the basis of *ace-2* DNA sequences they fall within the *C. p. pipiens* clade. Remarkably, novel mtDNA haplotypes were found in samples from Europe and North Africa that are related, but different to

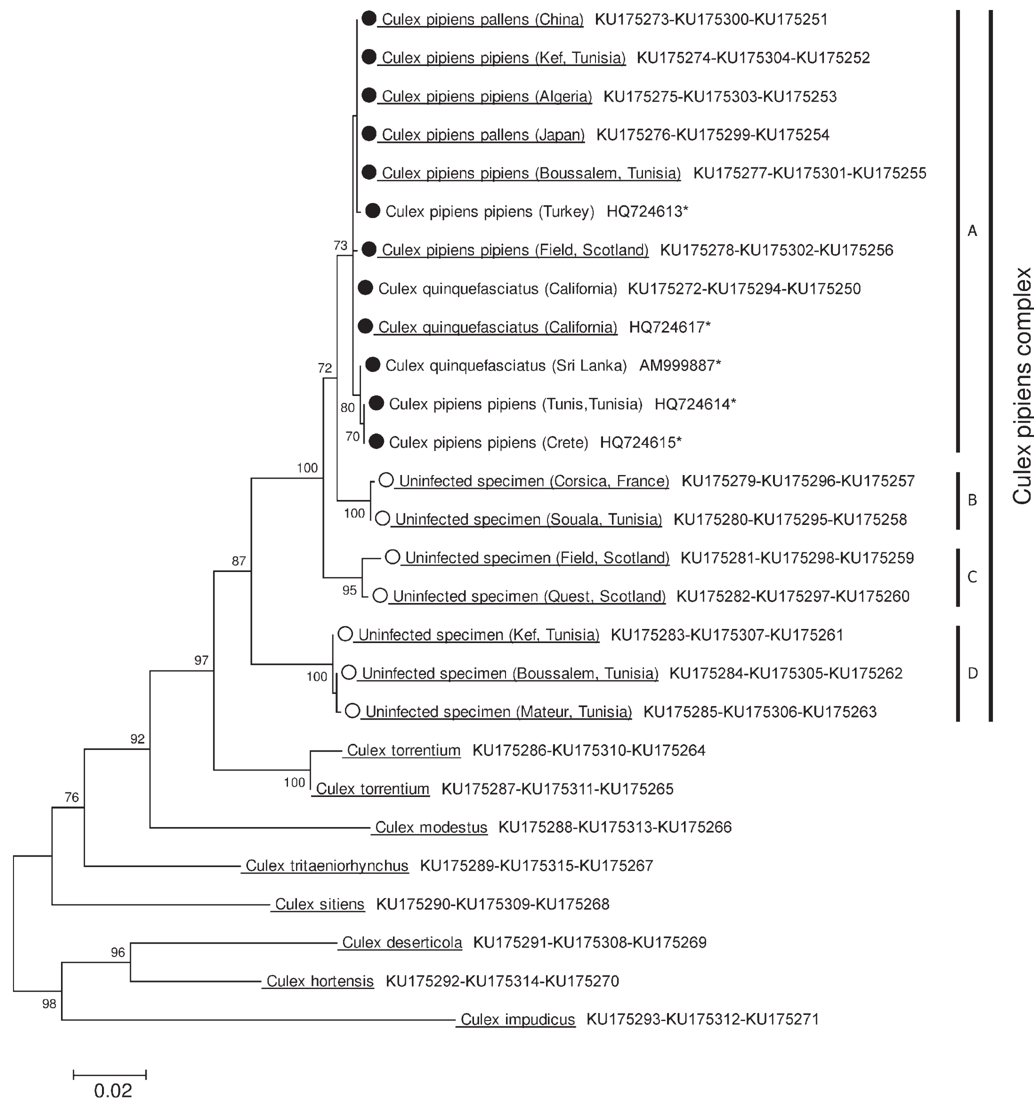

Figure 3. Mitochondrial phylogeny constructed using the maximum parsimony method based on concatenated sequences of nicotinamide adenine dinucleotide (*NADH*) *dehydrogenase subunit 2* (*ND2*), *ND4* and *cytochrome oxidase I* genes. White and black circles represent uninfected and *Wolbachia*-infected specimens from this study, respectively. Sequences from this study are underlined; other sequences are from GenBank (listed in Table S1). Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown. GenBank numbers are specified for each sample; asterisks represent accession numbers for the whole mitochondrial genome of that specimen.

the mtDNA haplotypes found in *Wolbachia*-infected *C. pipiens* complex members. This genetic pattern demonstrates that uninfected specimens are not a result of imperfect maternal transmission from *Wolbachia*-infected specimens but rather belong to a specific lineage. Our results along with those of Rasgon *et al*. (2006) thus corroborate the presence of a cryptic species within the *C. pipiens* complex, but we further evidence a far wider geographical distribution than previously suspected that ranges from northern Europe to South Africa.

Compelling evidence suggests that specimens of the cryptic species do not readily hybridize with *Wolbachia*-infected *C. pipiens* and *C. quinquefasciatus* specimens. The *C. pipiens* complex is formed by a group of evolutionarily closely related species that often hybridize, as shown between *C. pipiens* and *C. quinquefasciatus* in North America and Asia through both morphological and genetic analyses (Cornel *et al*., 2003; Fonseca *et al*., 2004, 2009). Variable levels of genetic isolation exist within the complex as shown between the two forms of *C. p. pipiens*, '*pipiens*' and '*molestus*': they are reproductively isolated in the north of Europe, whereas extensive hybridization is present in the south of Europe and the USA (Fonseca *et al*., 2004). However, the uninfected cryptic species seems clearly reproductively isolated from all the other complex members. As CI should

induce the rapid invasion of *Wolbachia*, no stable coexistence of infected and uninfected mosquitoes is expected within host populations (Engelstädter & Telschow, 2009); this is precisely the case within the *C. pipiens* and *C. quinquefasciatus* populations where *w*Pip infection is at fixation (Rasgon & Scott, 2003; Duron *et al*., 2005; Dumas *et al*., 2013). In Europe and North Africa, the presence of sympatric populations of the uninfected cryptic species and *Wolbachia*-infected *C. p. pipiens* thus suggests that the cryptic species is reproductively isolated from *C. p. pipiens*, preventing the interspecies spread of the infection through cytoplasmic introgression. Similarly, in South Africa, the coexistence with *Wolbachia*-infected *C. quinquefasciatus* since at least the 1970s shows that the cryptic species is also reproductively isolated from *C. quinquefasciatus* (Cornel *et al*., 2003; Rasgon *et al*., 2006). The lack of hybridization in South Africa is also supported by the fact that in that location, no hybrids were detected following comparisons of morphological characters and enzyme electrophoresis profiles (Jupp, 1978; Cornel *et al*., 2003). The nature of the mechanism responsible for reproductive isolation remains however to be determined. *Wolbachia* may partially contribute to this isolation because, through unidirectional CI, crosses between infected males and uninfected females should be infertile. However, in this case, the other direction of the cross remains fertile, suggesting that the reproductive isolation of the uninfected cryptic species may be actually driven by other mechanisms, such as behavioural isolation or hybrid inviability.

The main biological traits of the cryptic species are also almost entirely unknown – except for the absence of *Wolbachia* – but they probably show distinctive features. Each known member of the *C. pipiens* complex exhibits specific behavioural and physiological traits that greatly influence their respective distribution and abundance (Vinogradova, 2000; Farajollahi *et al*., 2011). The most obvious variable traits include larval habitat preference (underground hypogeous vs. above-ground epigeous, rural vs. urban), vertebrate feeding pattern (mammals vs. birds), mating behaviour (eurygamy vs. stenogamy), gonotrophic development (autogeny vs. anautogeny) and ability of adult females to enter into hibernation (quiescence vs. diapause). Even the most closely related members of the complex differ dramatically in ecology, as best illustrated with the '*pipiens*' and '*molestus*' forms of *C. p. pipiens*: whereas the former is a bird-dependent anautogeneous mosquito (a bloodmeal is required for egg development) that diapauses during winter and needs open space to mate (eurygamy), the latter is rather adapted to environments associated with human activity (ie mammal-dependence, autogeny, lack of diapause and of stenogamy) (Vinogradova, 2000; Farajollahi *et al*., 2011). In this context, some observations about the cryptic species are worthy of note. First, we collected here the larvae of uninfected specimens in the same epigeous sites as *C. p. pipiens*; this suggests that both species may share the same ecological requirements at the larval stage. Second, Rasgon *et al*. (2006) collected wild gravid and recently blood-fed uninfected females resting inside geese and chicken coops in South Africa; this indicates that the cryptic species may bite birds, at least occasionally. Third, Jupp (1978) reported that SAP females (that is, the cryptic species) appear to be incapable of true diapause during winter in contrast to *C. p. pipiens* females from the Northern Hemisphere. This suggests that the cryptic species may develop continuous cohorts across the seasons, although lower temperatures should slow down development. Lastly, Jupp (1978) also reported eurygamous behaviour (the need for large open spaces for mating) of SAP specimens during laboratory assays, which suggests that the cryptic species may have evolved a complex nuptial flight, a feature also observed in some European populations of *C. p. pipiens* (Vinogradova, 2000; Farajollahi *et al*., 2011). Unfortunately, this eurygamous behaviour also limited further investigations on the cryptic species: because of the need for large open spaces for mating, females remain unfertilized in breeding cages and this prevented both the maintenance of a lab colony over generations and crossing experiments with other members of the *C. pipiens* complex (Jupp, 1978). Hence, the cryptic species may exhibit a singular combination of biological features that deserves to be further explored by other ways than lab rearing, such as field studies or population genetics investigations.

Another question remains concerning the risk of disease transmission to vertebrates by the cryptic species. Mosquitoes of the *C. pipiens* complex are well known to be major vectors of several human pathogens, including West Nile virus, St Louis encephalitis virus, and filarial worms, as well as of wildlife pathogens such as the avian malaria parasite (reviewed in Farajollahi *et al*. 2011). The cryptic species may thus transmit some of these pathogens depending on its specific physiological and behavioural traits, such as feeding preference. For example, a mixed feeding pattern, with females feeding both on mammals and birds, may transmit pathogens from a variety of avian hosts to humans, as observed with the West Nile virus in North American populations of *C. pipiens* (Kilpatrick *et al*., 2006; Hamer *et al*., 2008). In addition, the absence of *Wolbachia* in the cryptic species may also interfere drastically with the outcome of parasite infections (Moreira *et al*., 2009; Bian *et al*., 2010; Kambris *et al*., 2010; Dodson *et al*., 2014). In the *C. pipiens* complex, *Wolbachia* protects its hosts against

mortality induced by the avian malaria parasite *Plasmodium relictum* (Zélé *et al.*, 2012), but also increases its susceptibility to this pathogen, significantly increasing the prevalence of salivary gland stage infections (Zélé *et al.*, 2014). As both mosquito mortality and infection prevalence are two key determinants of epidemiology for many pathogens such as *Plasmodium*, these results suggest that the absence of *Wolbachia* in the cryptic species may drive singular vector competence.

On account of the distinct and coherent phylogenetic traits described above, we propose the designation '*Culex juppi* nov. sp.' for this *Culex* species, belonging to the *C. pipiens* complex and associated with the absence of *Wolbachia* infection. The specific name honours P. G. Jupp, who first described the absence of *Wolbachia* in a supposed *C. pipiens* population from South Africa (Jupp, 1978).

In conclusion, we confirm that a widespread cryptic species is present within the *C. pipiens* complex, in accordance with previous investigations. This raises a series of exciting questions related to both the main biological features of this cryptic species and the role of *Wolbachia* in the speciation process within a species complex. Future research is also needed to assess the potential of this cryptic species to vector pathogens relative to the other members of the *C. pipiens* complex.

Experimental procedures

Mosquito collection

Field *C. pipiens* larvae and pupae were collected in eight above-ground (epigeous) breeding sites in Europe (Scotland and Corsica) and North Africa (Tunisia) where we had preliminarily observed an unusual presence of uninfected specimens (Table 1). All specimens were stored in 70–95% ethanol at room temperature or in liquid nitrogen until examined for *Wolbachia* infection and DNA diversity.

To obtain additional DNA sequences for phylogenetic analyses, we also used collection specimens from the main taxa of the *C. pipiens* complex (*C. quinquefasciatus*, *C. p. pipiens*, including the '*pipiens*' and '*molestus*' forms, and *C. p. pallens*, which are all infected by *Wolbachia*) and from seven other *Culex* species (*Culex deserticola*, *Culex hortensis*, *Culex impudicus*, *Culex modestus*, *Culex sitiens*, *Culex torrentium* and *Culex tritaeniorhynchus*) (listed in Table S1).

Molecular typing

DNA was extracted from individual mosquitoes using a cetyltrimethyl-ammonium bromide protocol (Rogers & Bendich, 1988). The quality of mosquito DNA was systematically tested by PCR amplification of a conserved region of the mosquito *ace-2* acetylcholinesterase gene (Bourguet *et al.*, 1998). Worthy of note is that the *ace-2* primers used here (Table S2) are diagnostic of the *C. pipiens* complex: they are known to only amplify members of the *C. pipiens* complex and not other *Culex* species (Bourguet *et al.*, 1998; Smith & Fonseca, 2004).

The *Wolbachia* infections were next screened on the basis of two independent molecular assays, each using different pairs of primers (Table S2): PCR assays targeting the *Wolbachia wsp* surface protein gene (Zhou *et al.*, 1998) and PCR assays on the *w*Pip *ank2* gene, which encodes a protein with ankyrin motives (Duron *et al.*, 2007). Additionally, qPCR was performed to confirm the absence of *Wolbachia* infection in both negative *wsp* and *ank2* PCRs. Following Berticat *et al.* (2002), two qPCRs were performed on each mosquito's DNA: one was specific for the mosquito *ace-2* gene and the other was specific for the *Wolbachia wsp* gene. Assuming that these genes are present in a single copy per haploid genome of the host and the symbiont, the ratio between *wsp* and *ace-2* provides an estimation of the *Wolbachia* density in individual mosquitoes. Each DNA template was analysed in triplicate for *wsp* and *ace-2* qPCR quantification.

Mosquito DNA sequences were further obtained following PCR amplifications of two nuclear markers (*ace-2* and ITS2) and of three mtDNA genes (*ND2*, *ND4* and *COI*). PCR products of *ace-2*, *ND2*, *ND4* and *COI* were sequenced directly whereas PCR products of ITS2 were cloned (to separate the different copies present before sequencing) using a TOPO Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All fragments were next sequenced through both strands with an ABI Prism 310 sequencer using a BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA). Sequences were obtained from a subsample of seven uninfected specimens (one uninfected specimen was randomly sampled per breeding site exept from Zerga) and compared to sequences obtained from their sympatric infected counterparts. We also obtained additional sequences of *C. pipiens* members and of other *Culex* species either from molecular typing conducted in this study or directly from GenBank (detailed in Table S1). This includes the ITS2 and *ND4* sequences of SAP specimens available on GenBank (neither *ace-2*, *ND2* nor *COI* sequences from SAP specimens were obtained by previous studies).

Gene features and primers are listed in Table S2. All PCR cycle amplifications were conducted as follows: 5 min at 94 °C, followed by 30–40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 to 1.5 min depending on the fragment size. A QIAquick gel extraction kit (QIAGEN, Valencia, CA, USA) was used to purify the PCR products for sequencing. Sequences from *Culex* mtDNA and *ace-2* genes were obtained directly from purified products on an ABI Prism 3130 sequencer using a BigDye Terminator Kit (Applied Biosystems). For ITS2, purified PCR products were cloned into the TOPO-TA cloning vector (pCR 2.1-TOPO vector, Invitrogen), transformed into competent *Escherichia coli* cells (TOP10 Chemically Competent *E. coli*, Invitrogen), and further sequenced using the M13F primer. Chromatograms were checked and edited using CHROMAS LITE (http://www.technelysium.com.au), and sequence alignments were performed using CLUSTALW (Thompson *et al.*, 2002) implemented in MEGA (Kumar *et al.*, 2004). All new sequences have been deposited in the GenBank database (listed in Table S1).

Molecular and phylogenetic analyses

Statistical and phylogenetic analyses were carried out using the R statistical package (R Core Team, 2013) and the program MEGA (Kumar *et al.*, 2004), respectively. Phylogenetic relationships between infected and uninfected specimens were evaluated using nuclear ITS2 and *ace-2* sequences and mtDNA *ND2*, *ND4* and *COI* sequences. The GBLOCKS program (Castresana, 2000) with default parameters was used to remove poorly aligned positions and to obtain non-ambiguous sequence alignments. The evolutionary model most closely fitting the sequence data was determined using the Akaike information criterion. Phylogenetic analyses were based on ML analyses. A ML heuristic search, using a starting tree obtained by neighbour-joining, was conducted. Clade robustness was assessed by bootstrap analysis using 1000 replicates. POPART software (http://popart.otago.ac.nz) was used for inferring and visualizing mitochondrial haplotype relationships amongst populations using the minimum spanning network approach.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of mosquito samples and GenBank accession numbers used in this study. Underlined accession numbers represent new sequence data generated from this study.

Table S2. Genes and primers for screening and sequencing.

Figure S1. Nicotinamide adenine dinucleotide (NADH) dehydrogenase *dehydrogenase subunit 4* phylogenetic tree constructed using the maximum parsimony method. White and black circles represent uninfected and *Wolbachia*-infected specimens from this study, respectively. Sequences from this study are underlined; other sequences are from GenBank (listed in Table S1). Note that the uninfected specimens from Boussalem, Kef and Mateur that cluster with the southern African *Culex pipiens* specimens belong to the D clade as shown in Fig. 3. Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown.

Figure S2. *NADH dehydrogenase subunit 2* phylogenetic tree constructed using the maximum parsimony method. White and black circles represent uninfected and *Wolbachia*-infected specimens from this study, respectively. Sequences from this study are underlined; other sequences are from GenBank (listed in Table S1). Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown.

Figure S3. *Cytochrome oxidase I* phylogenetic tree constructed using the maximum parsimony method. White and black circles represent uninfected and *Wolbachia*-infected specimens from this study, respectively. Sequences from this study are underlined; other sequences are from GenBank (listed in Table S1). Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown.

Figure S4. Mitochondrial haplotype network constructed using the minimum spanning method based on concatenated sequences of *NADH dehydrogenase subunit 2* (*ND2*), *ND4* and *cytochrome oxidase I* genes. Uninfected specimens are underlined. Numbers on connecting lines are the number of nucleotide changes separating each haplotype.