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 *Culex pipiens* and *Culex quinquefasciatus*, both infected by the cytoplasmically inherited *Wolbachia* symbiont. A *Wolbachia*-uninfected population of *Culex pipiens* was however described in South Africa and was recently proposed to represent a cryptic species. In this study, we reconsider the existence of this species by undertaking an extensive screening for the presence of *Wolbachia*-uninfected *Culex 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 *Culex pipiens* complex. Remarkably, all uninfected specimens harbor mitochondrial haplotypes which 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 *Culex pipiens* complex members known to be infected by *Wolbachia*. Altogether, these results corroborate the presence of a widespread cryptic species within the *Culex pipiens*. The potential role of this cryptic *Culex* pipiens species in the transmission of pathogens remains however to be determined. The designation '*Culex juppi* nov. sp.' is proposed for this mosquito species.

Key words | *Wolbachia*, *Culex pipiens* mosquito complex, cytoplasmic incompatibility, mitochondria.

Introduction

Cytoplasmically inherited symbionts are widespread in arthropods (Duron *et al.* 2008a; Duron *et al.* 2008b; Weinert *et al.* 2007; Werren and Windsor, 2000). 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 (Cordaux *et al.* 2011; Engelstadter and Hurst, 2009; Werren *et al.* 2008). In many host species, *Wolbachia* has evolved a conditional sterility phenotype, known as cytoplasmic incompatibility (CI) (Engelstadter and Telschow, 2009; Werren *et al.* 2008). 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) produced viable progeny, that is unidirectional CI. This phenomenon provides a reproductive advantage to infected females and favors the *Wolbachia* spread 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 *Wolbachia* spread is also influenced by antagonist forces, such as an infection cost imposed on female hosts and imperfect transmission of *Wolbachia* to the eggs (Engelstadter and Telschow, 2009; Hoffmann *et al.* 1990). Taken together, these parameters determine an invasion threshold for CI, that is an infection frequency below which *Wolbachia* becomes extinct and above which it invades, typically until fixation (Engelstadter and Telschow, 2009; Hoffmann *et al.* 1990).

Mosquitoes of the *Culex pipiens* complex are naturally infected by a variety of CI-inducing *Wolbachia* strains belonging to the *w*Pip clade (Atyame et al. 2011a; Duron et al. 2006b; Rasgon and Scott, 2003). This system is characterized by a rapid diversification of CI

69 determinants (Duron *et al.* 2012; Nor *et al.* 2013) that has led to an unrivalled variability of
70 crossing types, including uni- and bi-directionally incompatible *w*Pip strain types (Atyame *et*
71 *al.* 2011b; Atyame *et al.* 2014; Duron *et al.* 2006a; Guillemaud *et al.* 1997). The two most
72 widespread members of this species complex are the common house mosquito, *Cx. pipiens*,
73 and the southern house mosquito, *Cx. quinquefasciatus* (Farajollahi *et al.* 2011; Smith and
74 Fonseca, 2004; Vinogradova, 2000). The first one, *Cx. pipiens*, is common in temperate
75 regions and is subdivided in two subspecies, *Cx. p. pipiens* (Europe and North and South
76 Africa) and *Cx. p. pallens* (East Asia). In addition, two recognized forms, '*pipiens*' and
77 '*molestus*', are also encountered in *Cx. p. pipiens* in the Northern hemisphere. The second
78 species, *Cx. quinquefasciatus*, is rather found across the tropics and the lower latitudes of
79 temperate regions. Both species, including all the subspecies and forms, are infected by *w*Pip
80 with infection frequency near or at fixation in field populations (Dumas *et al.* 2013; Duron *et*
81 *al.* 2005; Rasgon and Scott, 2003). This infection pattern is well explained by the ability of
82 *w*Pip-infected males to induce complete CI with uninfected females, a near perfect maternal
83 transmission of infection and a reduced effect on female fecundity (Duron *et al.* 2006c;
84 Rasgon and Scott, 2003). Two other species are currently recognized within this complex, but
85 they remain poorly studied: *Cx. australicus* and *Cx. globocoxitus*, which are both restricted to
86 Australia (Farajollahi *et al.* 2011; Smith and Fonseca, 2004) and are not infected by
87 *Wolbachia* (Irving-Bell, 1974).
88
89 The pattern of mitochondrial DNA (mtDNA) variation within the *Cx. pipiens* complex is
90 known to be confounded by the spread of *Wolbachia*: both are linked through maternal co-
91 transmission within egg cytoplasm, resulting in complete linkage disequilibrium of mtDNA
92 with *w*Pip infection (Atyame *et al.* 2011a; Dumas *et al.* 2013; Rasgon *et al.* 2006). The
93 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 (Atyame *et al.* 2011a; Dumas *et al.* 2013; Rasgon *et al.* 2006). Furthermore, occasional hybridization events have resulted in cytoplasmic introgression of both *w*Pip and associated mtDNA between *Cx. pipiens* and *Cx. 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 and 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 (Atyame *et al.* 2011a; Dumas *et al.* 2013; Rasgon *et al.* 2006).

Forty years ago, Irving-Bell (1977) reported the absence of *Wolbachia* in southern African *Cx. 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 morphologically indistinguishable from the *Wolbachia*-infected *Cx. pipiens* found in the Northern Hemisphere, and reproductively isolated from sympatric *Cx. quinquefasciatus* infected populations (Cornel *et al.* 2003; Jupp, 1978). Rasgon *et al.* (2006) have further characterized a higher mtDNA haplotype diversity in the SAP population relative to other populations of the *Cx. pipiens* complex. It was thus hypothesized that the uninfected SAP population may represent a cryptic species within the *Cx. pipiens* complex where *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 geographic record of this cryptic species.

Here, we reconsider the existence of cryptic species in the *Cx. pipiens* complex by (i) undertaking an extensive screening for the presence of *Wolbachia*-uninfected *Cx. pipiens* specimens, (ii) characterizing nuclear and mtDNA lineages of uninfected specimens through a multi-locus typing scheme and (iii) 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.

Material and methods

Mosquito collection

Field *Cx. pipiens* larvae and pupae were collected in 8 above-ground (epigeous) breeding sites in Europe (Scotland and Corsica) and North Africa (Tunisia) where we have preliminarily observed an unusual presence of uninfected specimens (Table 1). All specimens were further 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 *Cx. pipiens* complex (*Cx. quinquefasciatus*, *Cx. p. pipiens*, including the '*pipiens*' and '*molestus*' forms, and *Cx. p. pallens* that are all infected by *Wolbachia*) and from seven other *Culex* species (*Cx. deserticola*, *Cx. hortensis*, *Cx. impudicus*, *Cx. modestus*, *Cx. sitiens*, *Cx. torrentium* and *Cx. tritaeniorhynchus*) (listed in Table S1).

Molecular typing

DNA was extracted from individual mosquitoes using a Cetyl-Trimethyl-Ammonium Bromide (CTAB) protocol (Rogers and Bendich, 1988). The quality of mosquito DNA was systematically tested by PCR amplification of a conserved region of the mosquito *ace-2*

143 acetylcholinesterase gene (Bourguet *et al.* 1998). Worthy of note is that the *ace-2* primers
144 used here (Table S2) are diagnostic to the *Cx. pipiens* complex: they are known to only
145 amplify members of the *Cx. pipiens* complex and not other *Culex* species (Bourguet *et al.*
146 1998; Smith and Fonseca, 2004).
147
148 The *Wolbachia* infections were next screened on the basis of three independent molecular
149 assays, each using different pairs of primers (Table S2): PCR assays targeting the *Wolbachia*
150 *wsp* surface protein gene (Zhou *et al.* 1998) and PCR assays on the *w*Pip *ank2* gene which
151 encodes a protein with ankyrin motives (Duron *et al.* 2007). Additionally, real-time
152 quantitative PCR (qPCR) was performed to confirm the absence of *Wolbachia* infection in
153 both negative *wsp* and *ank2* PCR. According to Berticat *et al.* 2002, two qPCRs were
154 performed on each mosquito's DNA: one was specific for the mosquito *ace-2* gene and the
155 other was specific for the *Wolbachia wsp* gene. Assuming that these genes are present in a
156 single copy per haploid genome of the host and the symbiont, the ratio between *wsp* and *ace-2*
157 provided an estimation of the *Wolbachia* density in individual mosquitoes. Each DNA
158 template was analyzed in triplicate for *wsp* and *ace-2* qPCR quantification.
159
160 Mosquito DNA sequences were further obtained following PCR amplifications of two nuclear
161 markers (*ace-2* and the internal transcribed spacer 2 (ITS2)), and of three mtDNA genes (the
162 NADH Dehydrogenase Subunit 2 (*ND2*), the NADH Dehydrogenase Subunit 4 (*ND4*), and
163 the cytochrome oxidase I (*COI*)). PCR products of *ace-2*, *ND2*, *ND4* and *COI* were sequenced
164 directly while PCR products of ITS2 were cloned (to separate the different copies present
165 before sequencing), using the TOPO Cloning Kit (Invitrogen) according to the manufacturer's
166 instructions. All fragments were next sequenced through both strands with an ABI Prism 310
167 sequencer using the BigDye Terminator Kit (Applied Biosystems). Sequences were obtained

168 from a subsample of seven uninfected specimens (one uninfected specimen was randomly
169 sampled per breeding site) and compared to sequences obtained from their sympatric infected
170 counterparts. We also obtained additional sequences of *Cx. pipiens* members and of other
171 *Culex* species either from molecular typing conducted in this study or directly from GenBank
172 (detailed in Table S1). This includes the ITS2 and *ND4* sequences of SAP specimens
173 available on GenBank (neither *ace-2*, *ND2* nor *COI* sequences from SAP specimens were
174 obtained by previous studies).
175
176 Gene features and primers are listed in Table S2. All PCR cycle amplifications were
177 conducted as follows: 5 min at 94°C, followed by 30-40 cycles of 94°C for 30s, 50°C for 30s,
178 and 72°C for 1 to 1.5min depending on the fragment size. The QIAquick gel extraction kit
179 (QIAGEN, Valencia, CA) was used to purify the PCR products for sequencing. Sequences
180 from *Culex* mtDNA and *ace-2* genes were obtained directly from purified products on an ABI
181 Prism 3130 sequencer using the BigDye Terminator Kit (Applied Biosystems). For ITS2,
182 purified PCR products were cloned into the TOPO-TA cloning vector (pCR 2.1-TOPO vector,
183 Invitrogen), transformed into competent *Escherichia coli* cells (TOP10 Chemically
184 Competent *E. coli*, Invitrogen), and further sequenced using the M13F primer.
185 Chromatograms were checked and edited using CHROMAS LITE
186 (http://www.technelysium.com.au), and sequence alignments were performed using
187 CLUSTALW (Thompson *et al.* 2002) implemented in MEGA (Kumar *et al.* 2004). All new
188 sequences have been deposited in the GenBank database (listed in Table S1).
189
190 *Molecular and phylogenetic analyses*
191 Statistical and phylogenetic analyses were carried out using the R statistical package
192 (http://www.r-project.org/) 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 nonambiguous sequence alignments. The evolutionary model most closely fitting the sequence data was determined using Akaike information criterion. Phylogenetic analyses were based on maximum likelihood (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 among populations using minimum spanning network approach.

Results

Distribution of uninfected specimens

Three hundred and forty eight specimens from eight breeding sites located in Europe (2 sites in Scotland, UK, and one in Corsica, France) and North Africa (5 sites in Tunisia) were screened for the presence of *Wolbachia* (Table 1). Of the 348 specimens, 163 (47%) were found uninfected using the *Wolbachia wsp* and *ank2* PCR assays. The presence of both infected and uninfected specimens was further confirmed by qPCR assays: *Wolbachia* was then detected in four specimens previously diagnosed positive (on the basis of *wsp* and *ank2* PCR assays) but not in 11 other specimens previously diagnosed 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 between breeding sites as significant variation occurs between them (Fisher exact test, $P=2.10^{-16}$): uninfected specimens were more common in Tunisian breeding sites (158

uninfected specimens of 230 examined) than in European sites (5 of 118; Fisher exact test, $P=2.10^{-19}$).

Evolutionary origin of uninfected specimens

We further examined the evolutionary relationships of European and North African uninfected specimens with other members of the *Cx. pipiens* complex, including SAP, and with other *Culex* species (Table S1). We included in the phylogenetic analyses nuclear ITS2 and *ace-2* sequences (351 bp and 529 bp unambiguously aligned nucleotide sites, respectively) and mitochondrial *ND2* (329 bp), *ND4* (287 bp) and *COI* (450 bp) sequences. In total, 98 new sequences from nuclear and mitochondrial markers have been deposited on 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, ML analyses were all globally congruent with the current *Culex* classification: we recovered the clustering of *Cx. p. pipiens*, *Cx. p. pallens* and *Cx. quinquefasciatus* within the *Cx. pipiens* complex, the presence of *Cx. torrentium* as the closest relatives of the complex while other *Culex* species are more distantly related (Figures 1-3 and 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 *Cx. pipiens* members and all clearly fall within the complex as further detailed below. On the basis of ITS2 ML analysis, all *Cx. pipiens* complex members cluster with uninfected specimens, a pattern highlighting their common evolutionary origin (Figure 1). The ITS2 sequences however exhibit insufficient polymorphism between infected and uninfected specimens, preventing characterization of a clear genetic structure within the *Cx. pipiens* complex. In contrast, the *ace-2* sequences are more polymorphic between *Cx.*

pipiens complex members and the ML analysis is thus more discriminative. It clearly separates *Cx. p. pipiens* from *Cx. quinquefasciatus* and from *Cx. australicus* (Figure 2), as also observed in previous studies (Bourguet *et al.* 1998; Smith and Fonseca, 2004). Worthy of note is that the ML analysis recovered the clustering of the *Cx. quinquefasciatus* and *Cx. p. pallens ace-2* sequences; this was at first sight surprising, but is actually expected as hybridization occurs between these two taxa and the *Cx. quinquefasciatus ace-2* alleles are known to have widely introgressed within the *Cx. p. pallens* populations (Fonseca *et al.* 2009) resulting in the pattern observed in Figure 2. Remarkably, on the basis of *ace-2* sequences, all uninfected specimens cluster with *Cx. p. pipiens* specimens (which are *Wolbachia*-infected) and are thus more closely related to this subspecies than to any other members of the *Cx. pipiens* complex. None of the uninfected specimens we examined here is closely related to *Cx. australicus* (Figure 2), which is known to be not infected by *Wolbachia* (Irving-Bell, 1974).

Examination of each of the mtDNA markers (Figures S1, S2 and S3), as well as the *ND4*, *ND2* and *COI* concatenated set (Figure 3), unambigiously discriminates uninfected specimens from other *Cx. pipiens* complex members. The same *ND4* haplotype was observed among all the *Wolbachia*-infected *Cx. pipiens* members: *Cx. p. pipiens*, *Cx. p. pallens* and *Cx. quinquefasciatus* (Figure S1). By contrast, three distinct *ND4* haplotypes (93.0 to 97.9% of pairwise identity; differing by 6 to 20 positions on 287bp) were found in the European and North African uninfected specimens and none showed complete identity to the one present in *Wolbachia*-infected specimens (91.5 to 98.6% of pairwise identity between uninfected and *Wolbachia*-infected specimens; differing by 4 to 24 positions). Remarkably, one of the *ND4* haplotypes found in uninfected North African specimens shows complete identity with one SAP *ND4* haplotype (Figure S1). Similarly, *ND2* and *COI* sequences were also much more

variable among uninfected specimens than among infected members of the *Cx. pipiens* complex (Figures S2 and S3) although a comparison with SAP specimens was not possible (no SAP *ND2* and *CO1* sequences are available from previous studies since 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% of pairwise identity) specific to uninfected specimens (Figure 3). Furthermore, mitochondrial haplotype relationships analysis confirmed an unambiguous differentiation between uninfected specimens and other *Cx. pipiens* complex members (Figure S4).

Because occasional hybridization events have resulted in a *w*Pip-drive cytoplasmic introgression of associated mtDNA between *Cx. pipiens* and *Cx. quinquefasciatus* populations, these two species cannot be distinguished on the basis of their mtDNA sequences (Atyame *et al.* 2011a; Dumas *et al.* 2013). As a result of this global cytoplasmic homogenization, all the mtDNA sequences of infected mosquitoes cluster in a monophyletic subclade (Figures 3 and S1-S3). At least four main mtDNA subclades (labeled A-to-D hereafter) can be distinguished within the *Cx. pipiens* complex and it is obvious that this mtDNA structure mirrors the *Wolbachia* infection status: while the A subclade encompasses all mtDNA sequences of infected mosquitoes, the B, C and D subclades only contain mtDNA sequences of uninfected mosquitoes (Figure 3). The mtDNA diversity of the B, C and D subclades fits at least partially with geographic 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 *ND4* phylogeny, all the SAP specimens are more closely related to the uninfected specimens from Tunisia belonging to the D subclade (Figure S1).

Discussion

Our results illustrate the complexity of taxonomic relationships among members of the *Cx. 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 *Cx. pipiens* specimens in several breeding sites in Europe and North Africa. Using a multi-locus typing scheme, we further confirmed that these uninfected specimens unambiguously belong to the *Cx. pipiens* complex and on the basis of *ace-2* DNA sequences they fall within the *Cx. p. pipiens* clade. Remarkably, novel mtDNA haplotypes were found in samples from Europe and North Africa that are related, but different to the mtDNA haplotypes found in *Wolbachia*-infected *Cx. pipiens* complex members. This genetic pattern demonstrates that uninfected specimens are not due to 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 *Cx. pipiens* complex, but we further evidence a far wider geographic distribution than previously suspected that ranges from the Northern Europe to South Africa.

Compelling evidences suggest that specimens of the cryptic species do not readily hybridize with *Wolbachia*-infected *Cx. pipiens* and *Cx. quinquefasciatus* specimens. The *Cx. pipiens* complex is formed by a group of evolutionarily closely related species that often hybridize, as shown between *Cx. pipiens* and *Cx. quinquefasciatus* in North America and Asia through both morphological and genetic analyses (Cornel *et al.* 2003; Fonseca *et al.* 2004; Fonseca *et al.* 2009). Variable levels of genetic isolation actually exist within the complex as shown between the two forms '*pipiens*' and '*molestus*' of *Cx. p. pipiens*: they are reproductively isolated in the North of Europe, whereas extensive hybridization is present in the South of Europe and the United States (Fonseca *et al.* 2004). However, the uninfected cryptic species

seems clearly reproductively isolated from all the other complex members. Because CI should induce the rapid invasion of *Wolbachia*, no stable coexistence of infected and uninfected mosquitoes is expected within host populations (Engelstadter and Telschow, 2009); this is precisely the case within the *Cx. pipiens* and *Cx. quinquefasciatus* populations where *w*Pip infection is at fixation (Dumas *et al.* 2013; Duron *et al.* 2005; Rasgon and Scott, 2003). In Europe and North Africa, the presence of sympatric populations of the uninfected cryptic species and *Wolbachia*-infected *Cx. p. pipiens* thus suggests that the cryptic species is reproductively isolated from *Cx. p. pipiens*, preventing the inter-species spread of the infection through cytoplasmic introgression. Similarly, in South Africa, the co-existence with *Wolbachia*-infected *Cx. quinquefasciatus* since at least the 70's shows that the cryptic species is also reproductively isolated from *Cx. 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 (Cornel *et al.* 2003; Jupp, 1978). 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, the cross between infected males and uninfected females should be infertile. But, 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 behavioral 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 likely show distinctive features. Each known member of the *Cx. pipiens* complex exhibits specific behavioral and physiological traits that greatly influence their respective distribution and abundance (Farajollahi *et al.* 2011; Vinogradova,

2000). The most obvious variable traits include larval habitat preference (underground hypogeous *versus* above-ground epigeous, rural *versus* urban), vertebrate feeding pattern (mammals *versus* birds), mating behavior (eurygamy *versus* stenogamy), gonotrophic development (autogeny *versus* anautogeny) and ability of adult females to enter into hibernation (quiescence *versus* diapause). Even the most closely related members of the complex differ dramatically in ecology, as best illustrated with the '*pipiens*' and '*molestus*' forms of *Cx p. pipiens*: while the former is a bird-dependent anautogeneous mosquito (a blood meal 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 (i.e. mammal-dependence, autogeny, lack of diapause and stenogamy) (Farajollahi *et al.* 2011; Vinogradova, 2000). 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 than *Cx. 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 the *Cx. p. pipiens* females from 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 a eurygamous behavior (the need of large open space 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 *Cx. p. pipiens* (Farajollahi *et al.* 2011; Vinogradova, 2000). Unfortunately, this eurygamous behavior also limited further investigations on the cryptic species: because of the need of large open space

for mating, females remain unfertilized in breeding cages and this prevented to maintain a lab colony over generations and to conduct crossing experiments with other members of the *Cx. 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 as field studies or populations genetic investigations.

Another question remains concerning the risk of disease transmission to vertebrates by the cryptic species. Mosquitoes of the *Cx. 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 avian malaria parasite (reviewed in Farajollahi *et al.* 2011). The cryptic species may thus transmit some of pathogens depending on its specific physiological and behavioral traits, 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 *Cx. pipiens* (Hamer *et al.* 2008; Kilpatrick *et al.* 2006). In addition, the absence of *Wolbachia* in the cryptic species may also interfere drastically with the outcome of parasite infections (Bian *et al.* 2010; Dodson *et al.* 2014; Kambris *et al.* 2010; Moreira *et al.* 2009). In the *Cx. 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 as *Plasmodium*, these results suggest that the absence of *Wolbachia* in the cryptic species may drive singular vector competence relatively to the other members of the *Cx. pipiens* complex.

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

In conclusion, we confirm that a widespread cryptic species is present within the *Cx. pipiens* complex, in accordance with the 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 relatively to the other members of the *Cx. pipiens* complex.

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556 **Table 1.** List of mosquito breeding sites examined in this study. n, number of specimens; a,
557 *Wolbachia* infection status was diagnosed using both *wsp* and *ank2* PCR assays; b, *Wolbachia*
558 infection status was diagnosed in a subsample of specimens using qPCR assays.
559

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

560

Figure legend

Figure 1. Mosquito ITS2 phylogenetic tree constructed using 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. GeneBank numbers are specified for each sample.

Figure 2. Mosquito *ace-2* phylogenetic tree constructed using Maximum Parsimony method. Sequenced 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. GeneBank numbers are specified for each sample.

Figure 3. Mitochondrial phylogeny constructed using Maximum Parsimony method based on concatenated sequences of *ND2, ND4* and *COI* 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 TableS1). Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown. GeneBank numbers are specified for each sample, stars represent the accessions for whole mitochondrial genome.

Supplementary materials

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

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.

Culex taxa	Origin	Genebank accession numbers				
		ITS2	*ace-2*	*ND2*	*ND4*	*COI*
Culex pipiens complex						
Cx. australicus	Australia	-	AY497523	-	-	-
Cx. pipiens pipiens	Mateur, Tunisia	KU175318	KU175332	-	-	-
	Kef, Tunisia	KU175319	KU175335	KU175274	KU175304	KU175252
	Boussalem, Tunisia	KU175324	KU175334	KU175277	KU175301	KU175255
	Field, Scottland	KU175316	KU175333	KU175278	KU175302	KU175256
	Quest, Scottland	-	KU175336	-	-	-
	'Istanbul' lab strain from Istanbul, Turkey	-	-	HQ724613	HQ724613	HQ724613
	'Kol' lab strain from Kolymbari, Crete	-	-	HQ724615	HQ724615	HQ724615
	'Tunis' lab strain from Tunis, Tunisia	-	-	HQ724614	HQ724614	HQ724614
	'Harash' lab strain from Harash, Algeria	-	-	KU175275	KU175303	KU175253
	California	-	HQ881620, HQ881624, HQ881633	-	-	-
	Iran	EF539854-EF539855	-	-	-	-
	Russia	AJ850085	-	-	-	-
	Japan	-	AB294405	-	-	-
	Colorado	-	AY196910	-	-	-
	Iran	-	JF430595	-	-	-
Cx. pipiens pallens	Japan (1)	U33025-U33026	-	-	-	-
	Japan (2)	-	AB294404	-	-	-
	Japan (3)	-	-	KU175276	KU175299	KU175254
	Hangzhou, China	-	-	KU175273	KU175300	KU175251
Cx. quinquefasciatus	Johannesburg, South Africa	DQ341106-DQ341108, DQ341111-DQ341112	-	-	AY793691-AY793692	-
	Kenya	Z48468	-	-	-	-
	Belize	U22124	-	-	-	-
	Bangladesh	-	FJ416016, FJ416019	-	-	-
	Florida	-	AY196911	-	-	-
	'Pel' lab strain from Sri Lanka	-	-	AM999887	AM999887	AM999887
	'Slab' lab strain from California	-	-	HQ724617	HQ724617	HQ724617
	'MaClo' lab strain from California	-	-	KU175272	KU175294	KU175250
Uninfected *Cx. pipiens* specimens	Johannesburg, South Africa (SAP)	DQ341109-DQ341110, DQ341113-DQ341115	-	-	AY793694-AY793703	-
	Mateur, Tunisia	KU175323	KU175329	KU175285	KU175306	KU175263
	Kef, Tunisia	KU175322	KU175330	KU175283	KU175307	KU175261
	Boussalem, Tunisia	KU175325	KU175338	KU175284	KU175305	KU175262
	Souala, Tunisia	KU175321	KU175331	KU175280	KU175295	KU175258
	Field, Scottland	KU175317	KU175337	KU175281	KU175298	KU175259
	Quest, Scottland	KU175326	KU175327	KU175282	KU175297	KU175260
	Corsica, France	KU175320	KU175328	KU175279	KU175296	KU175257
Other *Culex* species						
Cx. chidesteri	Guatemala	GU562344	-	-	-	-
Cx. coronator	Mississippi	GU562346	-	-	-	-
Cx. deserticola	Tunisia	-	-	KU175291	KU175308	KU175269
Cx. hortensis	France	-	-	KU175292	KU175314	KU175270
Cx. impudicus	France	-	-	KU175293	KU175312	KU175271
Cx. interrogator	Guatemala	GU562345	-	-	-	-
Cx. modestus	France	-	-	KU175288	KU175313	KU175266
Cx. nigripalpus	Florida	GU562871	-	-	-	-
	Florida	-	AY196914	-	-	-
Cx. restuans	North America	U22137	-	-	-	-
	California	-	AY196912	-	-	-

Cx. salinarius	North America	U22142	-	-	-	-
	Florida	-	AY196913	-	-	-
Cx. sitiens	Juan de nova, Mozambic Chanel	-	-	KU175290	KU175309	KU175268
Cx. torrentium	Sweeden (1)	U33038, U33040	-	-	-	-
	Sweeden (2)	-	-	KU175286	KU175310	KU175264
	Scottland	-	-	KU175287	KU175311	KU175265
	England	-	AY497525	-	-	-
Cx. tritaeniorhynchus	Taiwan	U33041	-	-	-	-
	China	-	-	KU175289	KU175315	KU175267

Table S2. Genes and primers for screening and sequencing.

Organism	Gene	Product	Primers (5'-3')	Fragment size	Reference
Wolbachia					
	wsp	Wolbachia surface protein fragment	81F (TGGTCCAATAAGTGATGAAGAAAC)	81F-691R: 602 bp	(Braig, 1998)
			691R (AAAATTAAACGCTACTCCA)		
			wolpipdir (AGAATTGACGGCATTGAATA)	wolpipdir-wolpiprev: 151 bp	(Berticat, 2002)
			wolpiprev (CGTCGTTTTTGTTTAGTTGTG)		
	ank2	Ankyrin domain protein	ank2F (CTTCTTCTGTGAGTGTACGT)	313-511 bp	(Duron, 2007)
			ank2R2 (TCCATATCGATCTACTGCGT)		
Culex spp. (nuclear)					
	ace-2	Acetylcholinesterase	F1457 (GAGGAGATGTGGAATCCCAA)	F1457-B1246: 700 bp	(Bourguet, 1998)
			B1246 (TGGAGCCTCCTCTTCACGGC)		
			Acequantidir (GCAGCACCAGTCCAAGG)	Acequantidir-Acequantirev: 208 bp	(Berticat, 2002)
			Acequantirev (CTTCACGGCCGTTCAAGTAG)		
	ITS2	Internal transcribed spacer 2	ITS2F (ATGCTTAAATTTAGGGGGTAGTC)	514 bp	(Porter, 1991)
			ITS2R (ATCACTCGGCTCGTGGATCG)		
Culex spp. (mitochondrial)					
	ND2	NADH dehydrogenase subunit 2	ND2F2 (TCCCCCTAATAAATGAARGWAA)	451 bp	This study
			ND2R2 (GCTATTARTATTCAWCCTAART)		
	ND4	NADH dehydrogenase subunit 4	ND4F (GTTCATTTATGAYTACCWAA)	388 bp	This study
			ND4R (CTTCGTCTTCCTATTCGTTC)		
	COI	Cytochrome c oxidase 1	CO1CulexF (GTCAACCNGGDGTATTTATTGG)	558 bp	This study
			CO1CulexR (GGRTCTCCTCCTCCAATWGGRTC)		

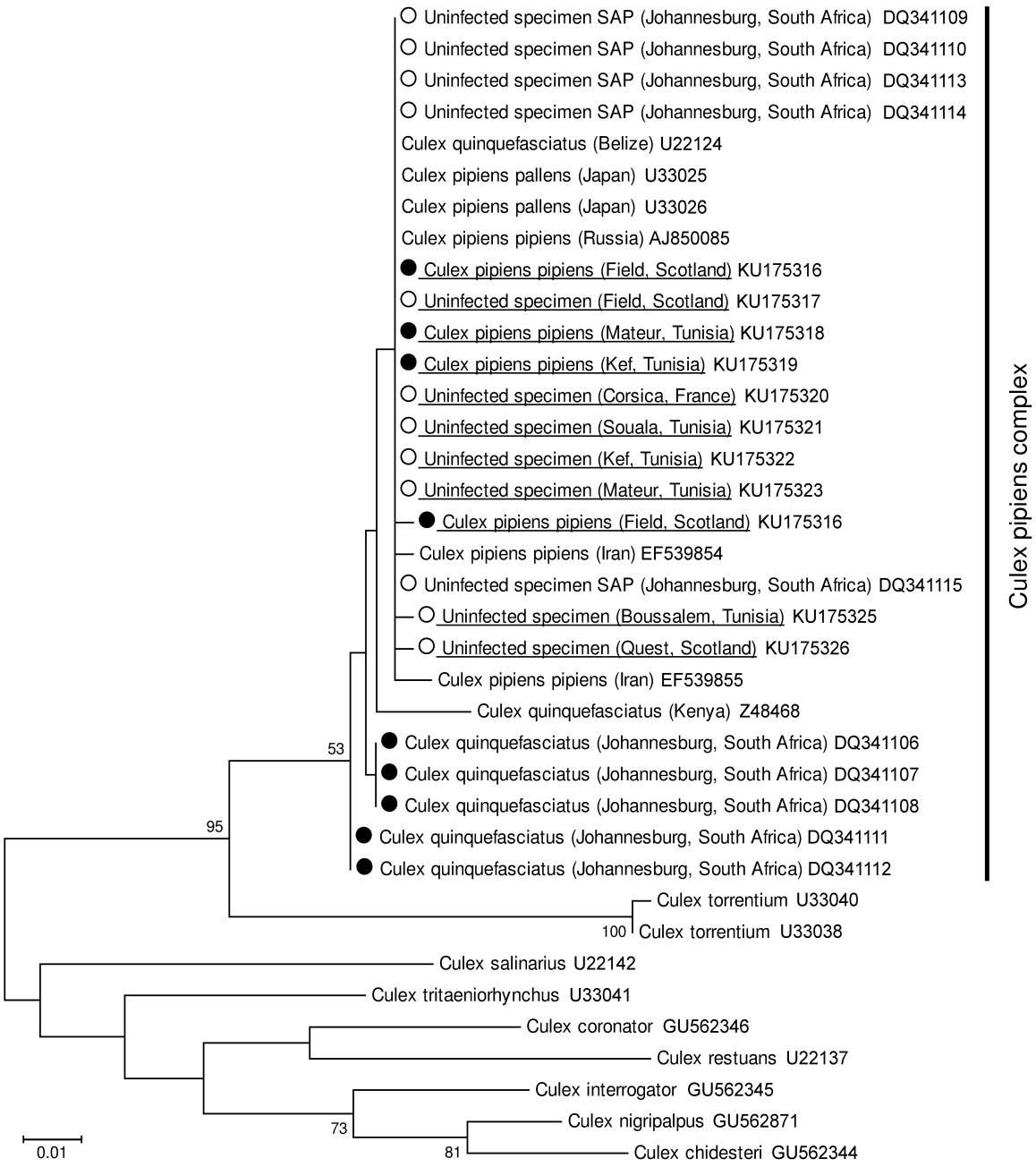
Figure legend

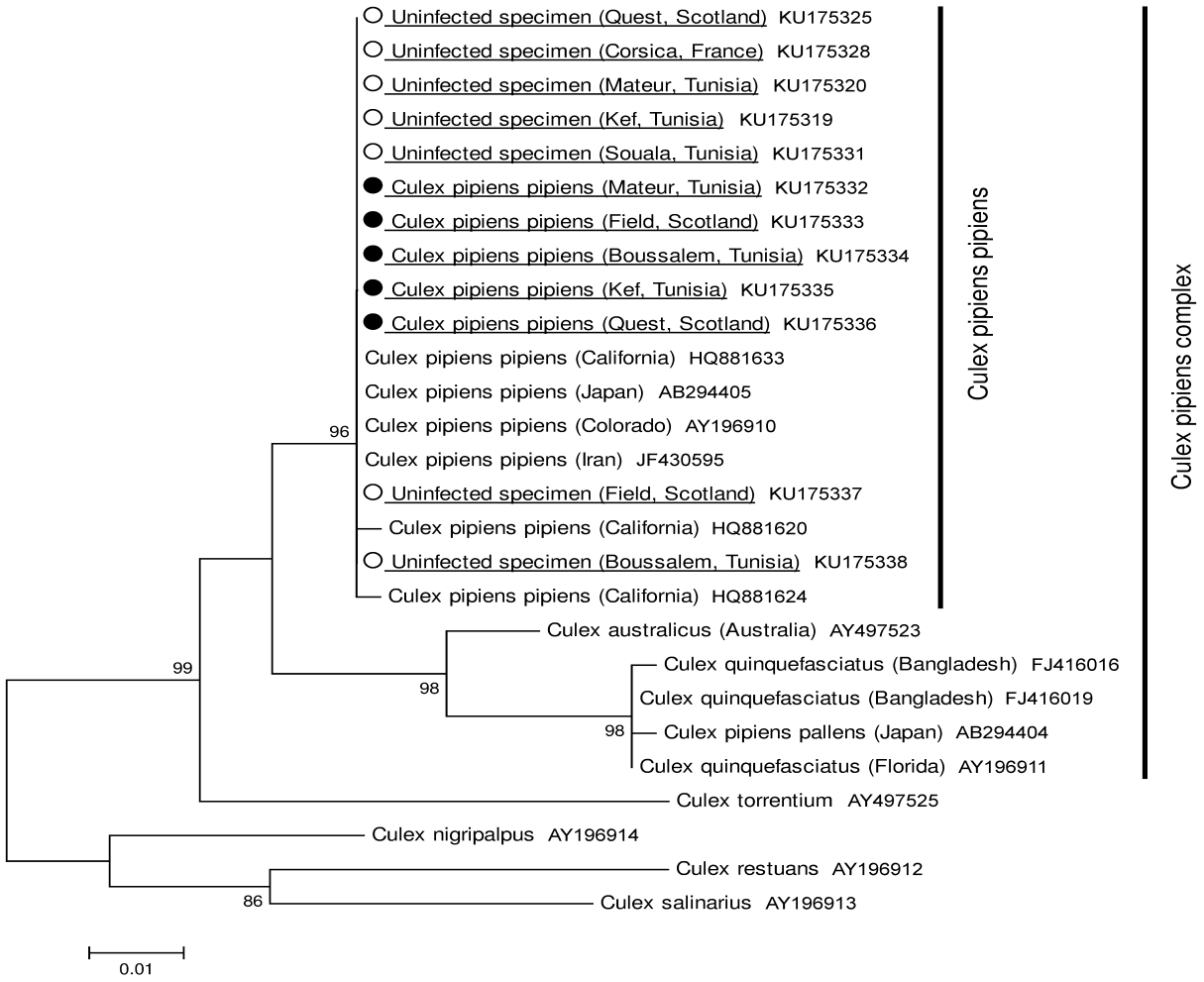
Figure S1. *ND4* phylogenetic tree constructed using Maximum Parsimony method. White and black circles represent uninfected and *Wolbachia*-infected specimens from this study, respectively. Sequenced from this study are underlined; other sequences are from Genbank (listed in TableS1). Note that the uninfected specimens from Boussalem, Kef and Mateur which cluster with the SAP specimens belong to the D clade as shown in Figure 3. Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown.

Figure S2. *ND2* phylogenetic tree constructed using Maximum Parsimony method. White and black circles represent uninfected and *Wolbachia*-infected specimens from this study, respectively. Sequenced from this study are underlined; other sequences are from Genbank (listed in TableS1). Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown.

Figure S3. *CO1* phylogenetic tree constructed using Maximum Parsimony method. White and black circles represent uninfected and *Wolbachia*-infected specimens from this study, respectively. Sequenced from this study are underlined; other sequences are from Genbank (listed in TableS1). Numbers on branches indicate percentage bootstrap support (500 replicates); only values above 70 are shown.

Figure S4. Mitochondrial haplotype network constructed using minimum spanning method based on concatenated sequences of *ND2*, *ND4* and *COI* genes. Uninfected specimens are underlined. Numbers on connecting lines are the number of nucleotide changes separating each haplotype.


Uninfected specimen SAP (Johannesburg, South Africa) DQ341109
Uninfected specimen SAP (Johannesburg, South Africa) DQ341110
Uninfected specimen SAP (Johannesburg, South Africa) DQ341113
Uninfected specimen SAP (Johannesburg, South Africa) DQ341114
Culex quinquefasciatus (Belize) U22124
Culex pipiens pallens (Japan) U33025
Culex pipiens pallens (Japan) U33026
Culex pipiens pipiens (Russia) AJ850085
Culex pipiens pipiens (Field, Scotland) KU175316
Uninfected specimen (Field, Scotland) KU175317
Culex pipiens pipiens (Mateur, Tunisia) KU175318
Culex pipiens pipiens (Kef, Tunisia) KU175319
Uninfected specimen (Corsica, France) KU175320
Uninfected specimen (Souala, Tunisia) KU175321
Uninfected specimen (Kef, Tunisia) KU175322
Uninfected specimen (Mateur, Tunisia) KU175323
Culex pipiens pipiens (Field, Scotland) KU175316
Culex pipiens pipiens (Iran) EF539854
Uninfected specimen SAP (Johannesburg, South Africa) DQ341115
Uninfected specimen (Boussalem, Tunisia) KU175325
Uninfected specimen (Quest, Scotland) KU175326
Culex pipiens pipiens (Iran) EF539855
Culex quinquefasciatus (Kenya) Z48468
Culex quinquefasciatus (Johannesburg, South Africa) DQ341106
Culex quinquefasciatus (Johannesburg, South Africa) DQ341107
Culex quinquefasciatus (Johannesburg, South Africa) DQ341108
Culex quinquefasciatus (Johannesburg, South Africa) DQ341111
Culex quinquefasciatus (Johannesburg, South Africa) DQ341112
Culex pipiens complex
53
95
100
Culex torrentium U33040
Culex torrentium U33038
Culex salinarius U22142
Culex tritaeniorhynchus U33041
Culex coronator GU562346
Culex restuans U22137
Culex interrogator GU562345
73
Culex nigripalpus GU562871
81
Culex chidesteri GU562344
0.01


Uninfected specimen (Quest, Scotland) KU175325
Uninfected specimen (Corsica, France) KU175328
Uninfected specimen (Mateur, Tunisia) KU175320
Uninfected specimen (Kef, Tunisia) KU175319
Uninfected specimen (Souala, Tunisia) KU175331
Culex pipiens pipiens (Mateur, Tunisia) KU175332
Culex pipiens pipiens (Field, Scotland) KU175333
Culex pipiens pipiens (Boussalem, Tunisia) KU175334
Culex pipiens pipiens (Kef, Tunisia) KU175335
Culex pipiens pipiens (Quest, Scotland) KU175336
Culex pipiens pipiens (California) HQ881633
Culex pipiens pipiens (Japan) AB294405
Culex pipiens pipiens (Colorado) AY196910
Culex pipiens pipiens (Iran) JF430595
Uninfected specimen (Field, Scotland) KU175337
Culex pipiens pipiens (California) HQ881620
Uninfected specimen (Boussalem, Tunisia) KU175338
Culex pipiens pipiens (California) HQ881624
Culex australicus (Australia) AY497523
Culex quinquefasciatus (Bangladesh) FJ416016
Culex quinquefasciatus (Bangladesh) FJ416019
Culex pipiens pallens (Japan) AB294404
Culex quinquefasciatus (Florida) AY196911
Culex torrentium AY497525
Culex nigripalpus AY196914
Culex restuans AY196912
Culex salinarius AY196913
96
99
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98
86
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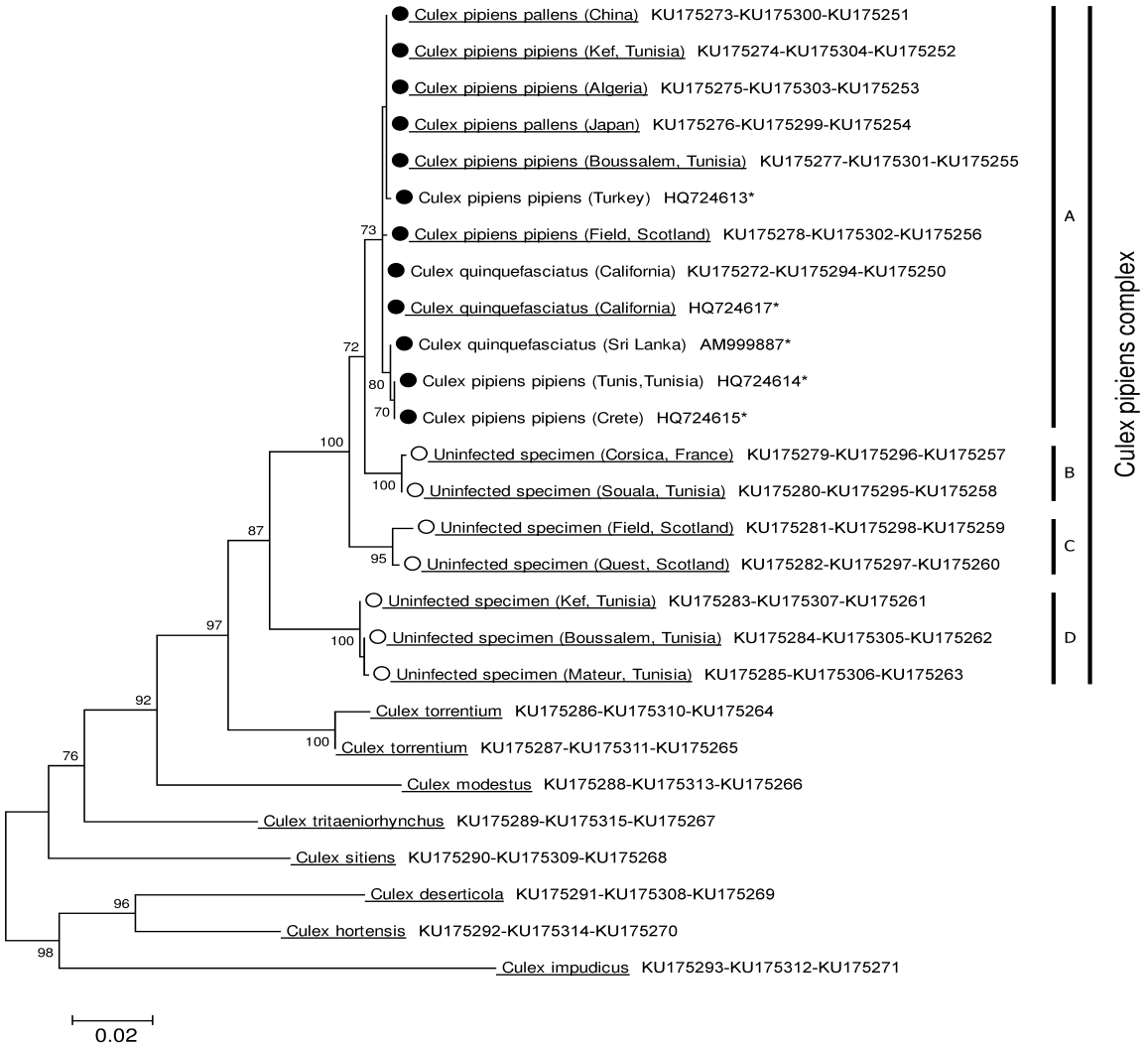
Culex pipiens pipiens
Culex pipiens complex


Culex pipiens pallens (China) KU175273-KU175300-KU175251
Culex pipiens pipiens (Kef, Tunisia) KU175274-KU175304-KU175252
Culex pipiens pipiens (Algeria) KU175275-KU175303-KU175253
Culex pipiens pallens (Japan) KU175276-KU175299-KU175254
Culex pipiens pipiens (Boussalem, Tunisia) KU175277-KU175301-KU175255
Culex pipiens pipiens (Turkey) HQ724613*
Culex pipiens pipiens (Field, Scotland) KU175278-KU175302-KU175256
Culex quinquefasciatus (California) KU175272-KU175294-KU175250
Culex quinquefasciatus (California) HQ724617*
Culex quinquefasciatus (Sri Lanka) AM999887*
Culex pipiens pipiens (Tunis,Tunisia) HQ724614*
Culex pipiens pipiens (Crete) HQ724615*
Uninfected specimen (Corsica, France) KU175279-KU175296-KU175257
Uninfected specimen (Souala, Tunisia) KU175280-KU175295-KU175258
Uninfected specimen (Field, Scotland) KU175281-KU175298-KU175259
Uninfected specimen (Quest, Scotland) KU175282-KU175297-KU175260
Uninfected specimen (Kef, Tunisia) KU175283-KU175307-KU175261
Uninfected specimen (Boussalem, Tunisia) KU175284-KU175305-KU175262
Uninfected specimen (Mateur, Tunisia) KU175285-KU175306-KU175263
Culex torrentium KU175286-KU175310-KU175264
Culex torrentium KU175287-KU175311-KU175265
Culex modestus KU175288-KU175313-KU175266
Culex tritaeniorhynchus KU175289-KU175315-KU175267
Culex sitiens KU175290-KU175309-KU175268
Culex deserticola KU175291-KU175308-KU175269
Culex hortensis KU175292-KU175314-KU175270
Culex impudicus KU175293-KU175312-KU175271
73
72
80
70
100
100
87
95
97
100
92
100
76
96
98
A
B
C
D
Culex pipiens complex
0.02

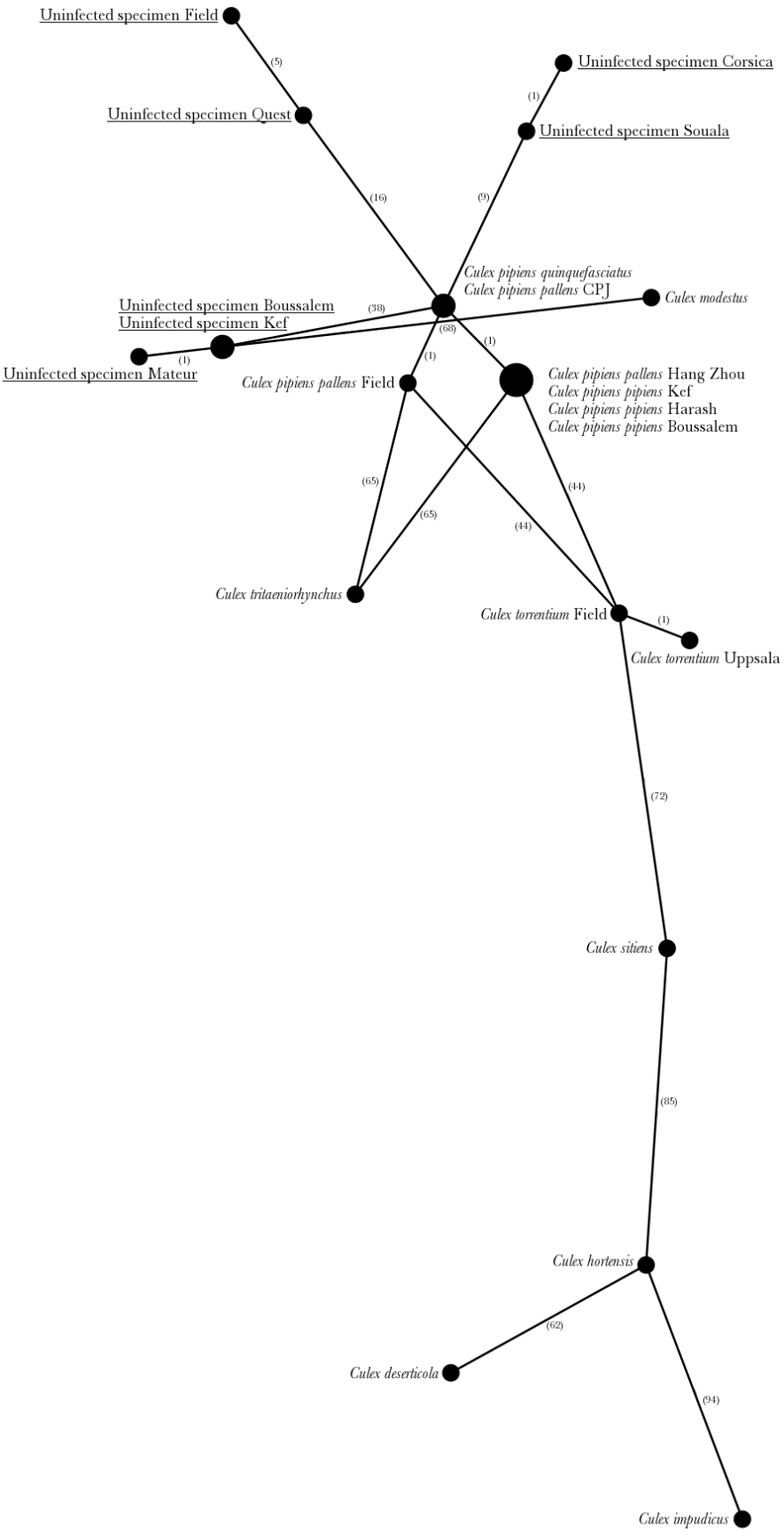

Uninfected specimen Field
(5)
Uninfected specimen Quest
(16)
Uninfected specimen Corsica
(1)
Uninfected specimen Souala
(9)
Culex pipiens quinquefasciatus
Culex pipiens pallens CPJ
Culex modestus
Uninfected specimen Boussalem
Uninfected specimen Kef
(38)
(68)
(1)
(1)
Uninfected specimen Mateur
Culex pipiens pallens Field
(1)
Culex pipiens pallens Hang Zhou
Culex pipiens pipiens Kef
Culex pipiens pipiens Harash
Culex pipiens pipiens Boussalem
(65)
(65)
(44)
(44)
Culex tritaeniorhynchus
Culex torrentium Field
(1)
Culex torrentium Uppsala
(72)
Culex sitiens
(85)
Culex hortensis
(62)
Culex deserticola
(94)
Culex impudicus

Figure S4