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1 **Diversification of *Wolbachia* endosymbiont in the *Culex pipiens***
2 **mosquito**

3

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20

21 **Abstract**

22 The α -proteobacteria *Wolbachia* are among the most common intracellular bacteria and have
23 recently emerged as important drivers of arthropod biology. *Wolbachia* commonly act as
24 reproductive parasites in arthropods by inducing cytoplasmic incompatibility (CI), a type of
25 conditional sterility between hosts harboring incompatible infections. In this study, we
26 examined the evolutionary histories of *Wolbachia* infections, known as *wPip*, in the common
27 house mosquito *Culex pipiens*, which exhibits the greatest variation in CI crossing patterns
28 observed in any insect. We first investigated a panel of twenty *wPip* strains for their genetic
29 diversity through a multi-locus scheme combining thirteen *Wolbachia* genes. Because
30 *Wolbachia* depend primarily on maternal transmission for spreading within arthropod
31 populations, we also studied the variability in the co-inherited *Cx. pipiens* mitochondria. In
32 total, we identified fourteen *wPip* haplotypes, which all share a monophyletic origin and
33 clearly cluster into five distinct *wPip* groups. The diversity of *Cx. pipiens* mitochondria was
34 extremely reduced, which is likely a consequence of cytoplasmic hitchhiking driven by a
35 unique and recent *Wolbachia* invasion. Phylogenetic evidence indicates that *wPip* infections
36 and mitochondrial DNA have co-diverged through stable co-transmission within the
37 cytoplasm and shows that a rapid diversification of *wPip* has occurred. The observed pattern
38 demonstrates that a considerable degree of *Wolbachia* diversity can evolve within a single
39 host species over short evolutionary periods. In addition, multiple signatures of recombination
40 were found in most *wPip* genomic regions, leading us to conclude that the mosaic nature of
41 *wPip* genomes may play a key role in their evolution.

42 **Introduction**

43 Arthropods are commonly infected by maternally transmitted endosymbionts. Although some
44 endosymbionts confer direct benefits to their hosts by providing anabolic functions or
45 resistance to pathogens (Haine 2008; Moran, McCutcheon, and Nakabachi 2008), others are
46 associated with alterations of host reproduction (Werren, Baldo, and Clark 2008; Engelstadter
47 and Hurst 2009). These symbionts represent reproductive parasites that include diverse
48 unrelated bacteria, among which the alpha-proteobacteria *Wolbachia* are the most widespread
49 (Duron et al. 2008; Hilgenboecker et al. 2008). In some host species, the successful spread of
50 *Wolbachia* is achieved by biasing the host's sex ratio toward the production of females (the
51 transmitting sex) through the induction of parthenogenesis, feminization or male-killing.
52 More commonly, *Wolbachia* are able to induce a form of conditional sterility, termed
53 cytoplasmic incompatibility (CI), between infected males and uninfected females or females
54 infected by incompatible strains (Werren, Baldo, and Clark 2008; Engelstadter and Hurst
55 2009). Such manipulations enable *Wolbachia* to spread through arthropod populations and
56 may drive arthropod evolution through their effects on host phenotypes (Moran, McCutcheon,
57 and Nakabachi 2008; Werren, Baldo, and Clark 2008; Engelstadter and Hurst 2009).

58

59 The dynamics of *Wolbachia* infections within the common house mosquito, *Culex pipiens*
60 complex, remain poorly understood. The most common members of the complex are the
61 subspecies *Cx. p. quinquefasciatus* (Say) and *Cx. p. pipiens* (L.), representing the southern
62 and northern mosquito populations, which are ubiquitous in tropical and temperate regions,
63 respectively (Barr 1982). Members of the *Cx. pipiens* complex exhibit the greatest variation
64 of CI crossing types observed in arthropods thus far (Laven 1967; O'Neill and Paterson 1992;
65 Guillemaud, Pasteur, and Rousset 1997; Duron et al. 2006; Atyame et al. in press). However,
66 an early genotyping approach using the *ftsZ* gene failed to reveal any polymorphism between

67 incompatible *Wolbachia* strains (Guillemaud, Pasteur, and Rousset 1997). Further analyses
68 used sequences of published complete *Wolbachia* genomes to characterize polymorphic
69 molecular markers. Genomes of *Wolbachia* strains infecting arthropod are scattered with
70 mobile genetic elements (MGE), such as prophages and transposable elements, which can
71 represent more than 20% of genome content (Wu et al. 2004; Klasson et al. 2008; Salzberg et
72 al. 2009; Klasson et al. 2009). Additionally, they contain an unusual high number of genes
73 encoding proteins with ankyrin (ANK) motif(s), which possibly mediate specific protein-
74 protein interactions (Sinkins et al. 2005; Duron et al. 2007; Walker et al. 2007). We further
75 developed genotyping approaches using ANK and MGE markers and used them to identify
76 more than 100 genetically distinct *Wolbachia* strains (referred to as *wPip* strains) in natural
77 populations of *Cx. pipiens* (Duron et al. 2005; Duron et al. 2006; Duron et al. 2007; Atyame
78 et al. in press; Duron, Raymond, and Weill in press).

79

80 In this study, we characterized the evolutionary history of *Wolbachia* infections in the *Cx.*
81 *pipiens* complex by examining the association between the *wPip* strains and *Cx. pipiens*
82 mitochondrial DNA variation. The predominant mode of *Wolbachia* transmission within a
83 species is vertical, *via* the egg cytoplasm (Werren, Baldo, and Clark 2008; Engelstadter and
84 Hurst 2009). Because *Wolbachia* and mitochondrial genomes are co-transmitted and,
85 therefore, in linkage disequilibrium, the spread of *Wolbachia* will strongly affects a host's
86 mtDNA diversity through indirect selection (review in Hurst and Jiggins 2005). However,
87 exceptions to strict vertical transmission have been found; in some cases, *Wolbachia* are also
88 transferred through horizontal transmission both within and among different host species,
89 although the mechanisms of transfer are not well understood (Ahrens and Shoemaker 2005;
90 Baldo et al. 2008; Raychoudhury et al. 2009). Consequently, the wide distribution of
91 *Wolbachia* among arthropods is generally assumed to result from complex interactions

92 between vertical and horizontal modes of transmission, modulated by their capacity to alter
93 host reproduction.
94
95 Here, we analyzed *wPip* variability and the associated mtDNA diversity in twenty *Cx. pipiens*
96 lines encompassing uni- and bidirectionally incompatible strains that originated from different
97 geographic areas. Our results showed that the *wPip* strains form a monophyletic clade of
98 closely related bacteria and that *Cx. pipiens* harbors a low level of mitochondrial variability,
99 which is a probable consequence of a recent *Wolbachia* invasion through cytoplasmic
100 hitchhiking. Investigation of *wPip* sequences revealed extensive recombination between *wPip*
101 strains, although multiple infections within a single mosquito were never detected using our
102 markers. However, a congruence between *wPip* and mtDNA phylogenies was shown,
103 demonstrating that *Wolbachia* mainly use maternal inheritance to spread through *Cx. pipiens*
104 populations. The evolutionary implications of horizontal transfers and the question of whether
105 the *Cx. pipiens* - *Wolbachia* association is a unique case or a representative example is
106 discussed.

107

108

109 **Materials and Methods**

110 **Mosquito collection**

111 Twenty *Cx. pipiens* lines from a broad geographical range were examined (Table 1). This
112 collection encompassed the two main *Cx. pipiens* subspecies, *Cx. p. pipiens* and *Cx. p.*
113 *quinquefasciatus*, which are naturally infected with compatible and incompatible *wPip* strains
114 (for more details, see Duron et al. 2006; Duron et al. 2007). The study also included the two
115 lines for which the *wPip* genome has been sequenced, *wPip*(Pel) (GenBank AM999887;
116 Klasson et al. 2008) and *wPip*(JHB) (ABZA01000000; Salzberg et al. 2009).

117

118 *Wolbachia* markers

119 The *wPip* strains were first genotyped for the five housekeeping genes developed for the
120 *Wolbachia* MultiLocus Strain Typing (MLST) methodology, *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*
121 (Baldo et al. 2006), and the *Wolbachia* surface protein gene *wsp* (Braig et al. 1998). The
122 MLST system is classically used to characterize the eight supergroups (A to I) currently
123 recognized within the *Wolbachia* genus (Lo et al. 2007; Ros et al. 2009). The polymorphism
124 of seven additional genes was also examined: the DNA mismatch repair protein gene *MutL*
125 (one copy in the *wPip*(Pel) genome), three ANK genes, *ank2* (one copy), *pk1* (three identical
126 copies), and *pk2* (two identical copies), and three phage genes, the methylase gene *GP12* (four
127 identical copies), the putative secreted protein gene *GP15* (also known as *VrlC*; one copy) and
128 the regulatory protein gene *RepA* (one copy). None of these genes was amplified from
129 *Wolbachia*-free *Cx. pipiens* lines, which confirmed their *Wolbachia* origin. A total of 13
130 *Wolbachia* genes, encompassing 19 distinct loci with a wide distribution along the *wPip*(Pel)
131 chromosome, were examined (Figure 1, Table S1).

132

133 *Culex pipiens* mitochondrial markers

134 The complete mitochondrial genome of the Pel line (15,587 bp) was obtained through BLAST
135 searches of the database of *wPip*(Pel) contig DNA sequences from the Wellcome Trust-
136 Sanger Institute website (http://www.sanger.ac.uk/Projects/W_pipientis/) using the
137 mitochondrial genome of *Aedes albopictus* (GenBank AY072044) as a probe. A similar
138 approach to obtain the mitochondrial sequences of the JHB line from the VectorBase website
139 (<http://www.vectorbase.org/>) showed several divergent mitochondrial sequences, some of
140 which exhibited low read coverage, making the JHB sequences unreliable for further analysis.

141

142 A set of primers (Table S2) was designed from the Pel sequences and further used to obtain
143 the complete mitochondrial genomes (with the exception of the A+T-rich region) of
144 additional *Cx. pipiens* lines. Specific primers were also used to amplify a 613 bp fragment
145 from the NADH dehydrogenase subunit 2 (*ND2*) gene, a 1,132 bp fragment from the NADH
146 dehydrogenase subunit 5 (*ND5*) gene and an 852 bp fragment from the cytochrome b (*cytb*)
147 gene from all the investigated *Cx. pipiens* lines (Table S2).

148

149 PCR amplification and sequencing

150 DNA was extracted from individual mosquitoes using a CetylTrimethylAmmonium Bromide
151 (CTAB) protocol (Rogers and Bendich 1988). Amplification conditions were 3 min at 94°C,
152 followed by 30 cycles of 94°C for 30 s, 52°C for 30 s (58°C for *MutL*), and 72°C for 1 to 1.5
153 min depending on the fragment size. Amplified fragments were run in agarose gel (1.5%)
154 electrophoresis. The QIAquick gel extraction kit (QIAGEN, Valencia, CA) was then used to
155 purify the PCR products. Sequences were obtained directly for purified products using an ABI
156 Prism 3130 sequencer with the BigDye Terminator Kit (Applied Biosystems). The sequences
157 have been deposited in the GenBank database (accession numbers in Tables S1 and S2).

158

159 Sequence analyses

160 Sequence alignments were carried out using CLUSTALW (Thompson, Gibson, and Higgins
161 2002) and corrected using MEGA (Tamura et al. 2007). The GBLOCKS program (Castresana
162 2000) with default parameters was used to remove poorly aligned positions and to obtain
163 nonambiguous sequence alignments. The number of variable sites (VI), nucleotide diversity
164 (π), G+C content and the ratios of nonsynonymous *versus* synonymous substitutions (Ka/Ks)
165 were computed using DNASP (Librado and Rozas 2009). Nonrandom associations between
166 each pair of loci were estimated through the measure of allele linkage disequilibrium (LD)

167 using the D' statistic (Lewontin 1964; Hedrick 1987). The exact test procedure implemented
168 in GENEPOP (Raymond and Rousset 1995) was further used to test LD significance.
169 Statistical analyses for intragenic recombination were performed with the Sawyer's test
170 implemented in GENECONV (Sawyer 1999). A Bonferonni adjustment correction for
171 multiple testing was applied (Hochberg 1988).

172

173 Annotation of the *Cx. pipiens* mitochondrial genome was based on alignments with
174 mitochondrial sequences from *Ae. albopictus* (AY072044), *Ae. aegypti* (EU352212),
175 *Anopheles gambiae* (L20934), *An. funestus* (DQ146364) and *An. quadrimaculatus* (L04272).

176

177 Tree reconstruction

178 Phylogenetic relationships were evaluated for *Wolbachia* and *Cx. pipiens* mitochondrial
179 sequences. The best-fitting models of sequence evolution for each dataset were determined
180 using the Akaike information criterion with MODELTEST v3.7 (Posada and Crandall 1998).

181 The selected model was the General Time Reversible model, with Gamma distributed among-
182 site rate variation (GTR+G) for both *Wolbachia* and mitochondrial sequence datasets.

183 Bayesian inferences (BI) were used to reconstruct phylogenies using Mr. Bayes v 3.1.2
184 (Ronquist and Huelsenbeck 2003). Two independent replicates of four Metropolis-coupled
185 Monte Carlo Markov chains were run for 2,000,000 generations with Model parameters and
186 trees sampled every 200 generations. Bayesian posterior probabilities were obtained from the
187 50% majority-rule consensus of the sampled trees after discarding the initial burn-in period.

188 The resulting phylogenetic trees were visualized and edited in MEGA (Tamura et al. 2007).

189

190 *Wolbachia* genes were also analyzed within a phylogenetic network framework to account for
191 potentially conflicting signals due to recombination (Fitch 1997). A phylogenetic network

192 was constructed based on uncorrected *P*-distances using the Neighbor-net method (Bryant and
193 Moulton 2004) implemented in SPLITSTREE (Huson and Bryant 2006). Neighbor-net is a
194 distance-based method to construct a network as a generalization of all possible phylogenetic
195 trees that can be reconstructed from conflicting signals in the data.

196

197 Assessing the maximum age of mitochondrial sweep

198 We used the mitochondria data to infer the maximum age of the *Wolbachia* infection in *Cx.*
199 *pipiens* following the method of Rich et al. (1998). This method assumes that selection only
200 occurs at the protein level and that DNA polymorphism in degenerate sites is neutral. We
201 used fourfold and twofold synonymous sites from protein coding mtDNA sequences to assess
202 the age of the sweep. The number of twofold and fourfold synonymous sites in each coding
203 region was computed with MEGA (Tamura et al. 2007), and a conservative Jukes-Cantor
204 correction was applied for multiple hits.

205

206

207 **Results**

208 Monophyletic origin of the *wPip* strains

209 The MLST and *wsp* genes did not exhibit sequence variation between the *wPip* strains (eight
210 strains were examined here, i.e. *wPip*(Sl), *wPip*(Tn), *wPip*(Ko), *wPip*(Lv), *wPip*(Is),
211 *wPip*(Mc), *wPip*(Pel) and *wPip*(JHB)), establishing that these strains are very closely related.

212 The *wPip* MLST sequence data were compared to sequences from 18 other strains belonging
213 to five distinct *Wolbachia* supergroups (A, B, D, F and H). The phylogenetic tree obtained
214 from the 2,079-bp concatenated MLST genes revealed that the *wPip* strains form a robust
215 monophyletic clade within the B supergroup, which is closely related to the *wBoll* strain
216 present in the butterfly *Hypolimnas bolina* (identity > 99.9%; Figure S1).

217

218 High variability of *wPip* genomes

219 Seven of the examined *Wolbachia* genes were polymorphic among the *wPip* genomes: the
220 DNA mismatch protein gene *MutL*, three ANK genes *ank2*, *pk1* and *pk2*, and three phage
221 genes *GP12*, *GP15* and *RepA*. Analyses revealed considerable allelic variability among the 20
222 *wPip* strains, with 2-8 alleles being found per gene (Table S3). This polymorphism was
223 mainly due to nucleotide substitutions, insertions or deletions (indels); note that an insertion
224 of the *Tr1* transposon (also known as ISWpi1; see Duron et al. 2005; Cordaux 2008) was
225 observed within the *RepA* sequence of three *wPip* strains. An additional source of variability
226 arose from a *GP15* deletion in the *wPip*(JHB) genome. A letter was attributed to each distinct
227 allele of the seven genes, the combination of which identified 14 *wPip* haplotypes among the
228 twenty strains (Table 2).

229

230 Although the prophage related genes *pk1*, *pk2* and *GP12* were found to be present in several
231 copies in the *wPip*(Pel) genome, divergent copies were never amplified from our *wPip* strains:
232 direct sequences of PCR products were easily readable and showed no overlapping peaks.
233 This indicates that the different copies (if any) present in each *wPip* strain examined here are
234 identical, as observed in *wPip*(Pel). It further shows that only mono-*wPip*-infections (or multi-
235 infections by closely related *wPip* strains) are present within *Cx. pipiens* individuals.

236

237 High recombination in *wPip* genomes

238 Recombination, both within and between *Wolbachia* genes, can blur molecular signals and
239 result in misleading observations related to strain relationships. For this reason, we checked
240 the possibility of recombination among the seven polymorphic markers obtained here (*MutL*,
241 *ank2*, *pk1*, *pk2*, *GP12*, *GP15* and *RepA*). Pairwise tests for intergenic recombination revealed

242 significant linkage disequilibrium (LD) for *MutL*, *ank2*, *pk1*, *GPI2*, and *GPI5* (Table S4).
243 Alleles at these five loci are not randomly associated and are stably co-transmitted within the
244 *wPip* chromosome. However, nonsignificant LD was found between *pk2* and *RepA* and
245 between these two genes and the other five genes, showing that recombination has disrupted
246 genome clonality by shuffling the *RepA* and *pk2* alleles among *wPip* strains. Intragenic
247 recombination was also detected for at least five genes by Sawyer's test (*MutL*, *pk1*, *pk2*,
248 *GPI2* and *GPI5*; Table S3). Intragenic recombination results in identical nucleotides or
249 amino acid motifs in *wPip* strains divergent at other loci, which are readily apparent through
250 the examination of sequence alignments (Figure S2).

251

252 Gene rearrangements in *wPip* genomes

253 Genome organization of *wPip* strains was analyzed by comparing the locations of the thirteen
254 genes surveyed in this study in the *wPip*(Pel) chromosome and in the five major *wPip*(JHB)
255 contigs presently available (Figure S3). There are several rearrangements distinguishing these
256 genomes, in which diverse genes have been inverted (e.g. *gatB*, *coxA*), translocated (*MutL*,
257 *RepA*), duplicated or deleted (three and one *pk1* copies are found in *wPip*(Pel) and *wPip*(JHB),
258 respectively). Notably, rearrangements are not limited to phage regions, which are prone to
259 movements within and between genomes, but also affect housekeeping genes.

260

261 Inference of *wPip* strain relationships

262 Phylogenetic analyses of the 20 *wPip* strains using the six *wPip* genes, *MutL*, *ank2*, *pk1*, *pk2*,
263 *GPI2* and *GPI5*, revealed significant topological incongruence, as expected for a dataset
264 affected by recombination (Figure S4). For instance, the *wPip*(S1), *wPip*(Bf-B) and *wPip*(Mc)
265 strains are genetically similar for four markers (*ank2*, *pk1*, *GPI2* and *GPI5*), but appear
266 distantly related for two others markers (*MutL* and *pk2*).

267

268 To assess *wPip* strain relationships, we performed phylogenetic analyses based on the
269 concatenated sequences of the seven genes. The concatenated tree deduced from Bayesian
270 inference splits the *wPip* clade into five groups (designated *wPip*-I to *wPip*-V; Figure 2).
271 However, recombination can create artificial grouping of *wPip* strains, and network analysis
272 was thus conducted to visualize recombination effects, which were illustrated by multiple
273 boxes (Figure 3). The evolutionary history of *wPip* strains appears as a complex network with
274 multiple pathways interconnecting strains, emphasizing the mosaic nature of *wPip* genomes.
275 Interestingly, despite recombination, the network analysis was congruent with the Bayesian
276 tree in recovering the same five *wPip* groups with strong bootstrap values.

277

278 A spatial structuring of *wPip* diversity emerged when the geographic distribution of *wPip*
279 groups was examined, despite the limited number (19) of strains. The most common group,
280 *wPip*-I, is distributed widely from Asia to Europe (Figure 4), and all *wPip* strains recently
281 identified at La Réunion island (Indian Ocean) by Atyame et al. (in press) belong to that
282 group. The *wPip*-V group is only found in East Asia, and the *wPip*-II and *wPip*-III groups
283 have an apparently discontinuous distribution, with strains being found in very distant
284 geographic areas (for example, the *wPip*-II strains are from Australia and Europe).

285

286 Low mitochondrial diversity in *Cx. pipiens*

287 *Cx. pipiens* Pel mitochondrial DNA exhibits classical features found in other mosquito
288 species that have been analyzed. It contains tightly packed genes with high A+T content
289 (78.2%). There are twenty-two genes coding tRNAs, two coding ribosomal RNAs, thirteen
290 genes coding subunits of enzymes involved in oxidative phosphorylation and, finally, an A+T
291 rich noncoding region (Figure S5 and Table S5). The genes are arranged along the

292 chromosome in a manner similar to that of other mosquito species (Beard, Mills Hamm, and
293 Collins 1993; Mitchell, Cockburn, and Seawright 1993; Krzywinski, Grushko, and Besansky
294 1997).

295

296 The complete *Cx. pipiens* mitochondrial genome (14,856 bp without the A+T rich region) was
297 sequenced from the lines Ko, Tn, Sl and Is and compared to the Pel genome. Overall, the five
298 mtDNA sequences displayed a very low variability, with only 36 variable nucleotidic
299 positions being found (ca. 2%), and two sequences were strictly identical (lines Ko and Tn).
300 Among the 13 protein-coding genes, five genes (*atp8*, *atp6*, *ND3*, *ND4L* and *ND6*) showed no
301 polymorphism, whereas *ND2*, *ND5* and *cytb* were the most polymorphic (Figure S5).

302

303 A likely explanation of the low mtDNA diversity in *Cx. pipiens* populations is that
304 cytoplasmic hitchhiking has occurred during *Wolbachia* invasion, as suggested earlier by
305 Guillemaud et al. (Guillemaud, Pasteur, and Rousset 1997) and Rasgon et al. (Rasgon,
306 Cornel, and Scott 2006). To confirm this hypothesis, we compared the nucleotide diversity
307 per site (π) at four mitochondrial loci in the eleven Culicidae species for which the presence
308 or absence of *Wolbachia* has been documented (Table 3 and supplementary materials). Only
309 two species, *Cx. pipiens* and *Ae. albopictus*, are known to be infected, whereas *Wolbachia*
310 infection was never found in the nine other species. These two *Wolbachia*-infected species
311 harbor significantly lower mtDNA diversity than the uninfected species (Wilcoxon test,
312 $W=10$, $P = 0.008$). For instance, the worldwide mtDNA diversity of *Cx. pipiens* is lower than
313 the diversity observed in the North American populations of *Cx. tarsalis*, an uninfected
314 species (Venkatesan et al. 2007). The low diversity of mtDNA observed in the *Cx. pipiens*
315 and *Ae. albopictus* populations led us to conclude that *Wolbachia* is most likely the causative
316 agent of mitochondrial sweeps in these taxa.

317

318 Recent mitochondrial sweep in *Cx. pipiens* complex

319 We then assessed the date of the mitochondrial sweep using the nucleotide divergence of 13
320 protein-coding mtDNA genes from the *Cx. pipiens* Is line and *Ae. albopictus* (GenBank
321 AY072044). We estimated the substitution rate for these genes at twofold and fourfold
322 degenerate sites with a conservative Jukes–Cantor correction. The genera *Culex* and *Aedes*
323 diverged approximately 172 to 226 My ago (Reidenbach et al. 2009). Using the most recent
324 estimate (172 My), the mtDNA substitution rates (substitution/site/year) were estimated at
325 5.1×10^{-8} and 19×10^{-8} for the twofold and fourfold degenerate sites, respectively, whereas,
326 when using the oldest estimate (226 My), the substitution rates were 39×10^{-9} and 15×10^{-8} .
327 Among the mitochondrial genomes of five *Cx. pipiens* lines (Is, Sl, Tn, Ko and Pel), we
328 observed 13 and 10 nucleotide differences among the twofold (n=2,938) and fourfold
329 (n=1,343) synonymous sites, respectively. Thus, this dates the *Cx. pipiens* mitochondrial
330 sweep between 12,000 and 16,000 years before present (95% confidence interval, if *Culex*
331 and *Aedes* diverged 172 My ago) or between 16,000 and 21,000 (95% confidence interval, if
332 the two genera diverged 226 My ago). It is possible that the date of the mitochondrial sweep
333 is even more recent as it was assumed here that the substitution rates are constant, an
334 assumption known to overestimate divergence times (Ho et al. 2005).

335

336

337 Clear co-divergence of *wPip* and *Cx. pipiens* mitochondria

338 The co-divergence of mitochondria and *wPip* was assessed by studying the sequences of the
339 three polymorphic *Cx. pipiens* mtDNA genes (*ND2*, *ND5* and *cytb*), encompassing 2,549 bp
340 (16.4% of the whole mitochondrial genome).

341

342 Analysis of the *Cx. pipiens* mtDNA sequences among the 19 lines indicated the presence of
343 14 haplotypes (named *pi1* to *pi14*), which differed overall at 22 variable nucleotide sites
344 (Table S6). The mtDNA of the *Cx. pipiens* lines differed by only one to nine nucleotides,
345 confirming their very high homology (99.6-99.9%). Phylogenetic analyses revealed two main
346 mitochondrial lineages (*pi1* to *pi5* and *pi6* to *pi14*) with strong branching support (Figure 5A).
347
348 The concatenated mtDNA phylogeny and the *wPip* phylogeny were congruent (Figure 5A and
349 B). A significant association was found between mtDNA haplotypes and *wPip* haplotypes
350 (Fisher's exact test, $P = 3 \times 10^{-5}$), as well as *wPip* groups ($P = 8 \times 10^{-4}$). This demonstrates that
351 *wPip* infections and mitochondrial DNA have co-diverged through stable co-transmission
352 within the cytoplasm in *Cx. pipiens* populations. Hence, the two main mitochondrial lineages
353 parallel the *wPip* divergence pattern and strongly confirm the *wPip* phylogeny. Additionally,
354 *Cx. pipiens* subspecies are not significantly associated with *wPip* haplotypes ($P = 0.37$), *wPip*
355 groups ($P = 0.26$) or mtDNA haplotypes ($P = 0.10$). Thus, *Cx. pipiens* nuclear genomes have
356 not co-diverged with mitochondria and *wPip* infections and exhibit a different evolutionary
357 history.

358

359

360 **Discussion**

361 Here, we examined 20 isolates of *Wolbachia* and their associated mitochondria within the *Cx.*
362 *pipiens* complex. The combined use of *Wolbachia* and host mtDNA multi-locus sequencing
363 revealed the processes driving the evolution of *Wolbachia* infections in this mosquito and
364 raised the question of their likeliness to occur in other *Wolbachia*-arthropod associations.

365

366 *Wolbachia* and mitochondrial markers reveal a recent diversification of *wPip* strains

367 The examined MLST and *wsp* markers showed that *wPip* infections form a robust
368 monophyletic clade within the B group of *Wolbachia*, confirming the results of Baldo et al.
369 (Baldo et al. 2006). Although these markers are widely used to characterize the genetic
370 diversity of *Wolbachia*, even within a host species (e.g. Dedeine et al. 2004; Baldo et al.
371 2006; Baldo et al. 2008; Raychoudhury et al. 2009), they displayed no variation among the
372 *wPip* infections in *Cx. pipiens*, which shows that *wPip* strains have a unique and recent
373 evolutionary origin. As observed in other *Wolbachia* infected species (Hurst and Jiggins
374 2005), mitochondrial diversity was low in populations of *Cx. pipiens* (and highly significantly
375 lower than in non-*Wolbachia*-infected mosquitoes), suggesting that *Wolbachia* have affected
376 mitochondrial polymorphism in this species through cytoplasmic hitchhiking. The observed
377 polymorphism of mitochondrial protein coding genes indicates that the mitochondrial sweep
378 due to the spread of *Wolbachia* occurred within the last 21,000 years. This dating is within the
379 range of values classically estimated for other *Wolbachia* host species of ca. < 100,000 years,
380 (Jiggins 2003; Keller et al. 2004; Duploux et al. 2010), and the evolutionary pathway of *wPip*
381 in *Cx. pipiens* could be similar to other *Wolbachia*/arthropod associations.

382

383 Multi-locus typing using seven *wPip* polymorphic markers, including domains of the MGE
384 and ANK genes, allowed the identification of 14 distinct *wPip* haplotypes, which cluster into
385 five distinct *wPip* groups. This typing approach also established that the two published *wPip*
386 genomes, *wPip*(Pel) and *wPip*(JHB), are genetically very close to each other compared to
387 strains belonging to other *wPip* groups, in spite of their genomic differences (Salzberg et al.
388 2009). The variability of the investigated mitochondrial markers corroborates the inferences
389 made from the *wPip* markers; thus, in *Cx. pipiens*, different mitochondrial haplotypes may
390 indicate that *wPip* infections are different. Overall, the observed genetic diversity indicates

391 that, after the spread of *Wolbachia*, diversification of *wPip* and *Cx. pipiens* mitochondria
392 occurred.

393

394 The diversity found for *wPip* exhibits geographic variations. A remarkable degree of diversity
395 was found in the Mediterranean area, where four of the five *wPip* groups are found, whereas a
396 reduced diversity was observed in other regions. The most common group, *wPip*-I, has a wide
397 distribution (Asia, Africa and Europe) and was also recently reported at La Réunion Island
398 (Indian Ocean) (Atyame et al. in press). In contrast, some *wPip* groups have a discontinuous
399 distribution, as exemplified by the *wPip*-II strains, which were found in Europe and in
400 Australia. Such a geographic pattern is likely to be a consequence of a recent worldwide
401 expansion due to human activity (Raymond et al. 1991; Fonseca et al. 2004; Fonseca et al.
402 2006) or/and to selective advantages, possibly including CI selection. However, the 20 *wPip*
403 infections investigated in this study represent a restricted sampling, occasionally from old
404 mosquito colonies, and further investigations are required to improve our knowledge of the
405 spatial structure of the *wPip* groups worldwide.

406

407 *wPip* strains are independent of *Cx. pipiens* subspecies

408 Strict vertical transmission must have favored the co-divergence of *wPip* and mtDNA within
409 shared cytoplasm. However, there was no clear association between *Cx. pipiens* subspecies
410 (nuclear diversity) and cytoplasmic diversity (i.e. *Wolbachia* and mtDNA): identical *wPip*
411 strains and identical mitochondrial haplotypes were found in the two subspecies, *Cx. p.*
412 *pipiens* and *Cx. p. quinquefasciatus*. A likely explanation for this is that the transfer of
413 cytoplasm between *Cx. pipiens* subspecies occurred through hybridization events, as observed
414 in *Drosophila* species (Rousset and Solignac 1995; Ballard 2000) and in butterfly species
415 (Jiggins 2003; Narita et al. 2006; Charlat et al. 2009). In *Cx. pipiens*, this hypothesis is well

416 supported by the many reports of genetic introgression between the two subspecies in areas
417 where they come into contact (Cornel et al. 2003; Fonseca et al. 2004). Hence, we can predict
418 that DNA barcoding programs using mtDNA will fail to discriminate between *Cx. p. pipiens*
419 and *Cx. p. quinquefasciatus*. Overall, these observations support the call of Hurst and Jiggins
420 (Hurst and Jiggins 2005) to not use mtDNA alone as a reliable means of taxa resolution.

421

422 Intense recombination impacts the structure of *wPip* genomes

423 The existence of extensive recombination among *wPip* strains sheds light on the mechanisms
424 shaping the evolution of *wPip* genomes since recombination can influence the adaptive
425 dynamics of *Wolbachia* by creating new alleles and thus allow the emergence of new
426 phenotypes. Recombination between distant *Wolbachia* genomes has been previously
427 documented (Jiggins et al. 2001; Bordenstein and Wernegreen 2004; Baldo, Lo, and Werren
428 2005; Gavotte et al. 2007), although in this study, we found recombination among very
429 closely related *Wolbachia* genomes. Evidence of recombination was found at almost all of the
430 examined *wPip* loci, WO-phage genes, as well as nonrelated phage loci. This shows that a
431 high level of gene flow occurs among the *Wolbachia* genomes in *Cx. pipiens*. Hence, the *wPip*
432 strains do not form a set of clones in which evolution is independent but, rather, represent a
433 large population of bacteria exchanging genetic information through lateral transfers.

434 Although no instances of multiple infection were detected using our markers, we must assume
435 that they occur, at least during a period long enough to allow recombination between strains.

436

437 Another consequence of recombination is that it can lead to misinterpretation of phylogenetic
438 relationships between strains. However, despite the extensive recombination observed, the
439 *wPip* and mitochondrial phylogenies are congruent: recombinations have not disrupted our
440 grasp of the evolutionary history of *wPip* strains, probably because the contribution of
441 recombinant regions in the phylogeny is weak compared to the diversity existing in

442 nonrecombinant DNA fragments. Therefore, as suggested by Baldo et al. (Baldo et al. 2006),
443 the use of a multi-locus approach, rather than single locus analysis, is required for a correct
444 understanding of the evolutionary history of *Wolbachia* infections.

445

446 The *Cx. pipiens*-*Wolbachia* association, a unique case?

447 The high number of *wPip* strains, which is still certainly underestimated, makes the *Cx.*
448 *pipiens* system remarkable because lower diversity is usually reported in *Wolbachia* of other
449 host species (e.g. Vavre et al. 1999; Mercot and Charlat 2004; Charlat et al. 2006; Arthofer et
450 al. 2009). However, it is possible that genetic variations of *Wolbachia* in other host species
451 could have been missed due to the methodology generally used to characterize these bacteria,
452 as it is generally assumed that a single *Wolbachia* strain is present within a host species when
453 the MLST or *wsp* markers are not variable. In *D. melanogaster*, a single strain, *wMel*, was
454 presumed to be present until Riegler et al. (Riegler et al. 2005) identified five distinct
455 genotypes by examining transposon insertion sites and chromosomal inversions. More recent
456 studies have reported different *Wolbachia* haplotypes solely on the basis of WO phage genes
457 in various host species, including crickets, beetles and butterflies (Charlat et al. 2009; Chafee
458 et al. 2010). Hence, the classical MLST system is well suited to characterize *Wolbachia*
459 belonging to distinct clades, but specific species typing systems based on markers with rapid
460 sequence evolution need to be developed to investigate the *Wolbachia* diversity that probably
461 exists in most associations.

462

463 Finally, the question remains of whether the *Cx. pipiens* - *Wolbachia* association is unique in
464 term of its extremely large CI diversity (e.g. Laven 1967; O'Neill and Paterson 1992;
465 Guillemaud, Pasteur, and Rousset 1997; Duron et al. 2006). We have clearly demonstrated
466 that the diversity of crossing types in this species is independent of nuclear backgrounds and

467 relies solely on *wPip* variability (Duron et al. 2006; Atyame et al. in press). The reason that a
468 similar CI system has not been reported in other *Wolbachia*-infected species remains a matter
469 for speculation, but it is possible that the crossing studies conducted in *Cx. pipiens* have been
470 more exhaustive than in any other species because of the intensive investigations that have
471 been carried out for clarifying its systematics and studying the inheritance of morphological
472 characters since the 1930s (e.g. Marshall and Staley 1937; Roubaud 1941; Laven 1958;
473 Rozeboom 1958; Laven 1967; Barr 1975; Narang and Seawright 1982; Irving-Bell 1983). As
474 a result, the high variability of CI crossing types was investigated much earlier than the
475 causative agent was identified by Yen and Barr in 1971. Comparatively little work on the
476 variability of the effects of *Wolbachia* infection has been conducted in most arthropods,
477 except in *Drosophila* species, such as *D. simulans*, where five distinct crossing types
478 associated with distinct *Wolbachia* infections have thus far been identified (for review see
479 Mercot and Charlat 2004). Therefore, the possibility of the existence of variable reproductive
480 phenotypes in other host species remains to be examined.

481
482 In conclusion, the use of multi-locus typing combining *Wolbachia* and mitochondrial markers
483 highlights the processes underlying the evolutionary dynamics of *wPip* infections. The
484 diversification inside the *wPip* clade shows that a considerable amount of *Wolbachia* diversity
485 can be generated within a single host species in a short period of time. Further investigations
486 should examine the roles of recombination and MGE in the adaptive capacities of *Wolbachia*.
487 In particular, this could explain rapid changes of interactions between *Wolbachia* and their
488 hosts (Weeks et al. 2007; Echaubard et al. 2010) and play a key role in the evolution of
489 phenotypes induced by *Wolbachia*. Finally, an important question now is to determine
490 whether the *Cx. pipiens* - *Wolbachia* association is a unique case or, rather, a representative
491 example.

492

493

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503

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Mosquito line	Abbreviation	<i>Wolbachia</i> strain	<i>Culex pipiens</i> subspecies	Origin	Year of collection	Reference
Pel	Pel	wPip(Pel)	<i>quinquefasciatus</i>	Sri Lanka	1984	(Klasson et al. 2008)
JHB	JHB	wPip(JHB)	<i>quinquefasciatus</i>	South Africa	2001	(Salzberg et al. 2009)
Manille-A	Ma-A	wPip(Ma-A)	<i>quinquefasciatus</i>	Philippines	2003	(Duron et al. 2006)
Manille-B	Ma-B	wPip(Ma-B)	<i>quinquefasciatus</i>	Philippines	2003	(Duron et al. 2006)
Kara-C	Ka-C	wPip(Ka-C)	<i>quinquefasciatus</i>	China	2003	(Duron, Fort, and Weill 2006)
MaClo	Mc	wPip(Mc)	<i>quinquefasciatus</i>	California	1984	(Duron et al. 2005)
Slab	Sl	wPip(Sl)	<i>quinquefasciatus</i>	California	1950	(Duron et al. 2005)
Cotonou-A	Cot-A	wPip(Cot-A)	<i>quinquefasciatus</i>	Benin	2005	This study
Cotonou-B	Cot-B	wPip(Cot-B)	<i>quinquefasciatus</i>	Benin	2005	This study
Australie	Au	wPip(Au)	hybrid (<i>quinquefasciatus/pipiens</i>)	Australia	2004	(Duron, Fort, and Weill 2006)
El Palmar-A	Ep-A	wPip(Ep-A)	<i>pipiens</i>	Spain	2005	(Duron et al. 2007)
El Palmar-B	Ep-B	wPip(Ep-B)	<i>pipiens</i>	Spain	2005	(Duron et al. 2007)
LaVar	Lv	wPip(Lv)	<i>pipiens</i>	France	2003	(Duron et al. 2005)
Bifa-A	Bf-A	wPip(Bf-A)	<i>pipiens</i>	France	2002	(Duron et al. 2006)
Bifa-B	Bf-B	wPip(Bf-B)	<i>pipiens</i>	France	2002	(Duron et al. 2006)
Kol	Ko	wPip(Ko)	<i>pipiens</i>	Crete	2002	(Duron, Fort, and Weill 2006)
Keo-A	Ke-A	wPip(Ke-A)	<i>pipiens</i>	Cyprus	2003	(Duron et al. 2006)
Keo-B	Ke-B	wPip(Ke-B)	<i>pipiens</i>	Cyprus	2003	(Duron et al. 2006)
Tunis	Tn	wPip(Tn)	<i>pipiens</i>	Tunisia	1992	(Duron et al. 2005)
Istanbul	Is	wPip(Is)	<i>pipiens</i>	Turkey	2003	(Duron et al. 2005)

759 **Table 1.** Description of *Culex pipiens* lines and wPip strains.

Strain	Gene							Haplotype
	<i>MutL</i>	<i>ank2</i>	<i>pk1</i>	<i>pk2</i>	<i>GP12</i>	<i>GP15</i>	<i>RepA</i>	
w Pip(Pel)	a	a	a	a	a	a	a	A
w Pip(Cot-A)	a	a	a	a	a	a	a	
w Pip(Cot-B)	a	a	a	a	a	a	a	
w Pip(Ko)	a	a	a	a	a	a	a	
w Pip(Tn)	a	a	a	a	a	a	a	
w Pip(Ma-B)	a	a	a	a	a	a	a	
w Pip(JHB)	a	a	a	a	a	-	a	B
w Pip(Ep-A)	a	a	a	d	a	a	a	C
w Pip(Ep-B)	a	a	a	d	a	a	a	
w Pip(Bf-A)	a	a	a	a	a	a	b	D
w Pip(Lv)	b	e	c	a	b	b	a	E
w Pip(Au)	d	e	c	a	b	f	a	F
w Pip(Ke-A)	c	e	c	a	d	e	a	G
w Pip(Ke-B)	c	e	c	a	e	e	a	H
w Pip(Sl)	e	b	b	b	b	c	b	I
w Pip(Bf-B)	e	b	b	c	b	c	b	J
w Pip(Mc)	b	b	b	b	b	c	b	K
w Pip(Is)	c	c	d	a	c	d	a	L
w Pip(Ka-C)	f	d	e	a	f	g	a	M
w Pip(Ma-A)	f	d	e	a	g	g	a	N

760

761

762 **Table 2.** Allelic profiles of the seven polymorphic wPip genes in 20 wPip strains. Letters A to

763 N represent the 14 wPip haplotypes. Dash indicates a gene deletion.

Taxon (subfamily, species)	π (no of sequences)				<i>Wolbachia</i> infection (reference)
	<i>cytb</i>	<i>ND4</i>	<i>COI</i>	<i>COII</i>	
Culicinae					
<i>Culex pipiens</i>	0.0021 (19)	0.0004 (14)	0.0000 (24)	0.0007 (8)	yes (Hertig and Wolbach 1924)
<i>Cx. tarsalis</i>	–	0.0116 (64)	–	–	no (Rasgon and Scott 2004)
<i>Culex</i> sp.	–	0.0090 (10)	–	–	no (Rasgon, Cornel, and Scott 2006)
<i>Aedes aegypti</i>	0.0094 (16)	0.0202 (46)	–	–	no (Kittayapong et al. 2000)
<i>Ae. albopictus</i>	0.0043 (14)	–	0.0039 (23)	–	yes (O'Neill et al. 1992)
<i>Ae. caspius</i>	–	–	0.0094 (7)	0.0063 (21)	no (Ricci et al. 2002)
<i>Ae. vexans</i>	–	–	0.0185 (7)	0.0084 (7)	no (Kittayapong et al. 2000; Ricci et al. 2002)
Anophelinae					
<i>Anopheles aconitus</i>	–	–	0.0053 (13)	0.0066 (35)	no (Kittayapong et al. 2000)
<i>An. funestus</i>	0.0066 (11)	–	–	–	no (Ricci et al. 2002)
<i>An. gambiae</i>	–	–	0.0053 (48)	–	no (Ricci et al. 2002)
<i>An. maculipennis</i>	–	–	0.0063 (62)	–	no (Ricci et al. 2002)

766 **Table 3.** Nucleotide diversity (π) of mitochondrial genes in eleven mosquito species (Culicidae).

767 **Figure legends**

768 **Figure1.** Map of the *wPip*(Pel) genome showing the position of the thirteen genes examined.
769 Number in brackets (1 to 4) indicates identical copies of a given gene located in different
770 positions along the chromosome. Black boxes indicate the locations of prophage regions; the
771 MLST and *wsp* genes are reported in grey.

772

773 **Figure 2.** Phylogenic tree of *wPip* strains obtained from concatenated data set (*MutL*, *ank2*,
774 *pk1*, *pk2*, *GP12*, *GP15* and *RepA* sequences) by Bayesian analysis. Posterior probabilities
775 obtained are shown at major nodes. The scale bar is in units of substitutions/site.

776

777 **Figure 3.** Network analysis obtained from concatenated data set (*MutL*, *ank2*, *pk1*, *pk2*,
778 *GP12*, *GP15* and *RepA* sequences) using the Neighbor-net method. Each edge (or a set of
779 parallel edges) corresponds to a split in the data set and has length equal to the weight of the
780 split. Incompatible splits, produced by recombination are represented by boxes in the
781 network. Only bootstrap values for major grouping are indicated. The five *wPip* groups
782 (highlighted) are connected by multiple pathways resulting from recombination between
783 *Wolbachia* genomes.

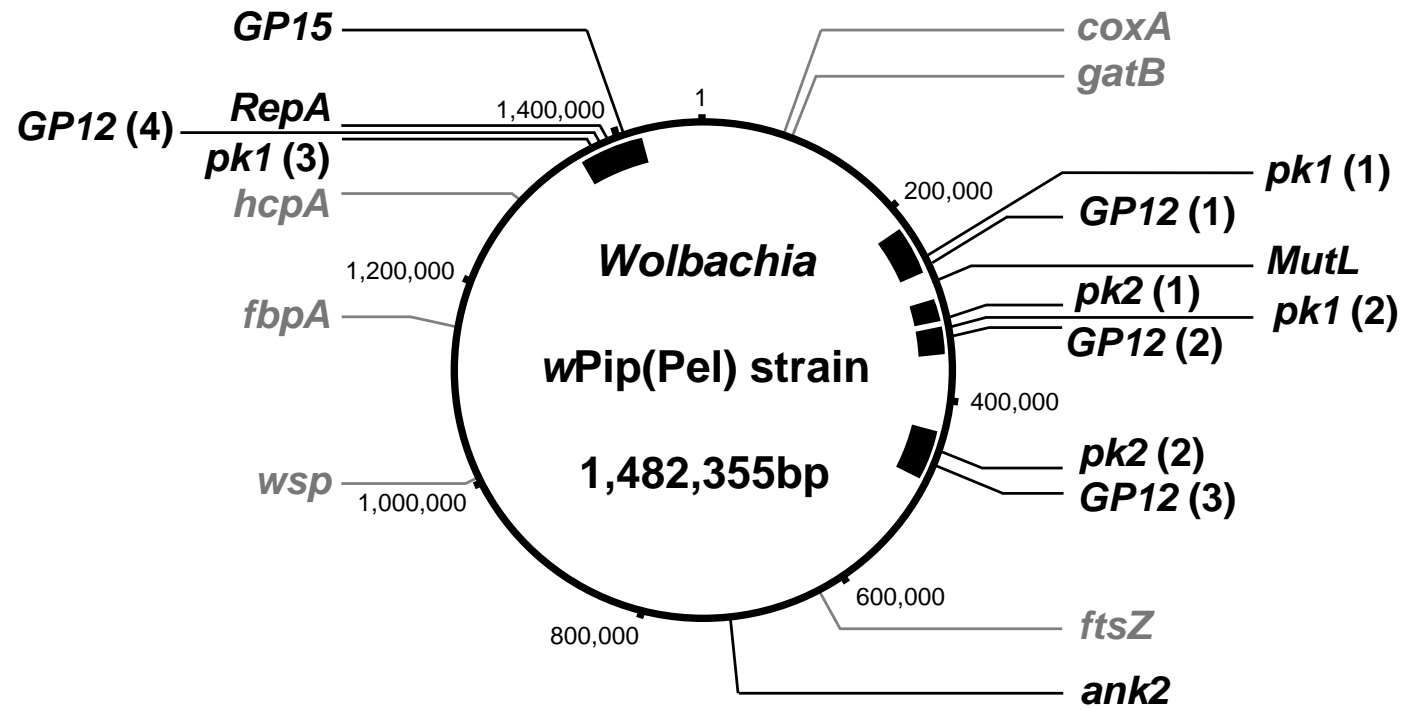
784

785 **Figure 4.** Distribution of *wPip* haplotypes and *wPip* groups in *Culex pipiens* populations.
786 Letters and symbols represent the *wPip* haplotypes and *wPip* groups, respectively. * *wPip*
787 haplotypes recently described by Atyame et al. (Atyame et al. in press).

788

789 **Figure 5.** Comparisons between phylogeny of *Culex pipiens* mitochondria and phylogeny of
790 the *wPip* strains. **A**, Mitochondrial phylogeny constructed using Bayesian inferences based on
791 *ND2*, *ND5* and *cytb* concatenated sequences. Names on branches indicate the mtDNA
792 haplotypes (*piI* to *piXIV*). **B**, *wPip* phylogeny obtained from concatenated data set (*MutL*,
793 *ank2*, *pk1*, *pk2*, *GP12*, *GP15* and *RepA* sequences). The five *wPip* groups are reported. The
794 scale bar is in units of substitutions/site.

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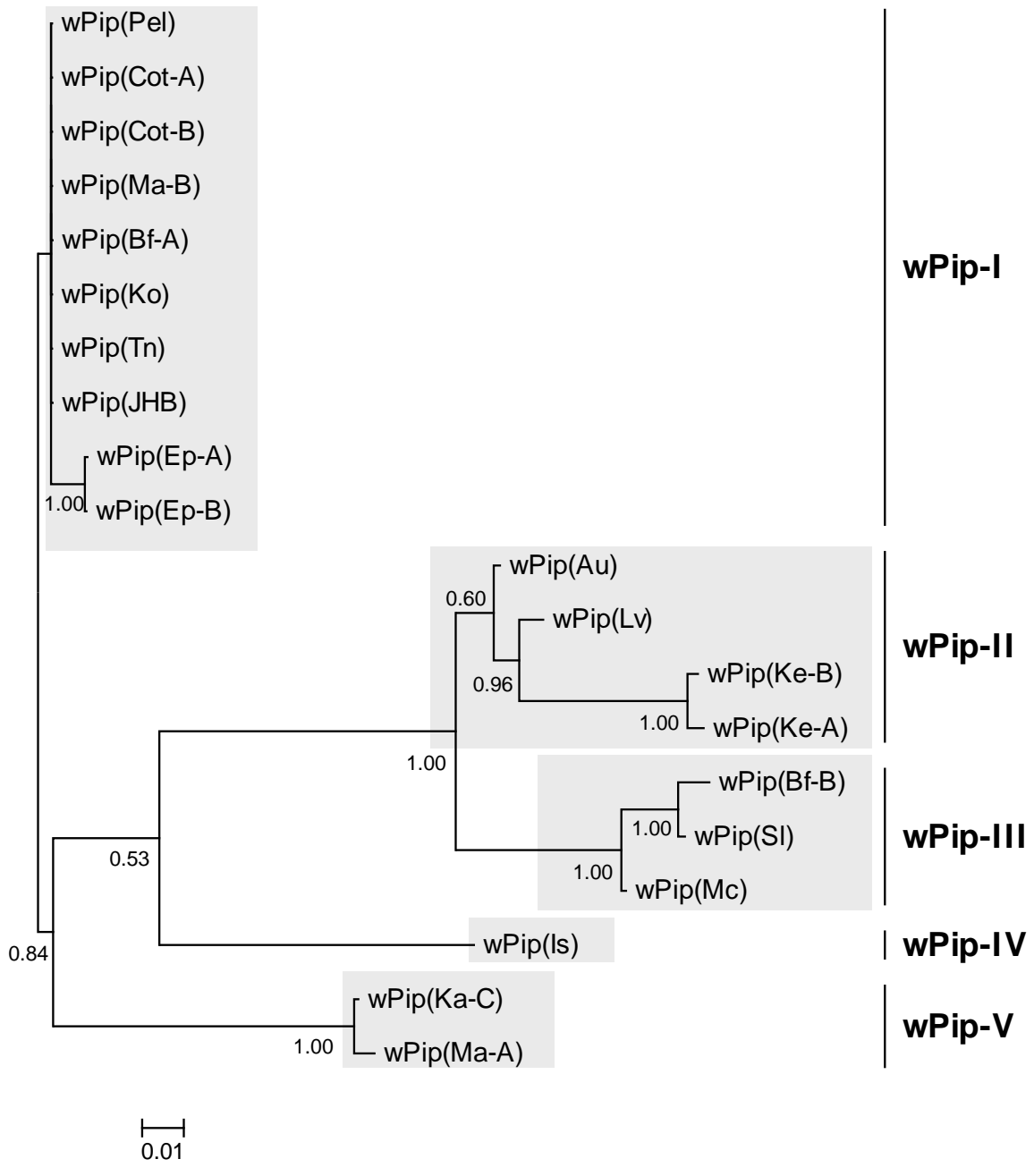


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Figure 1

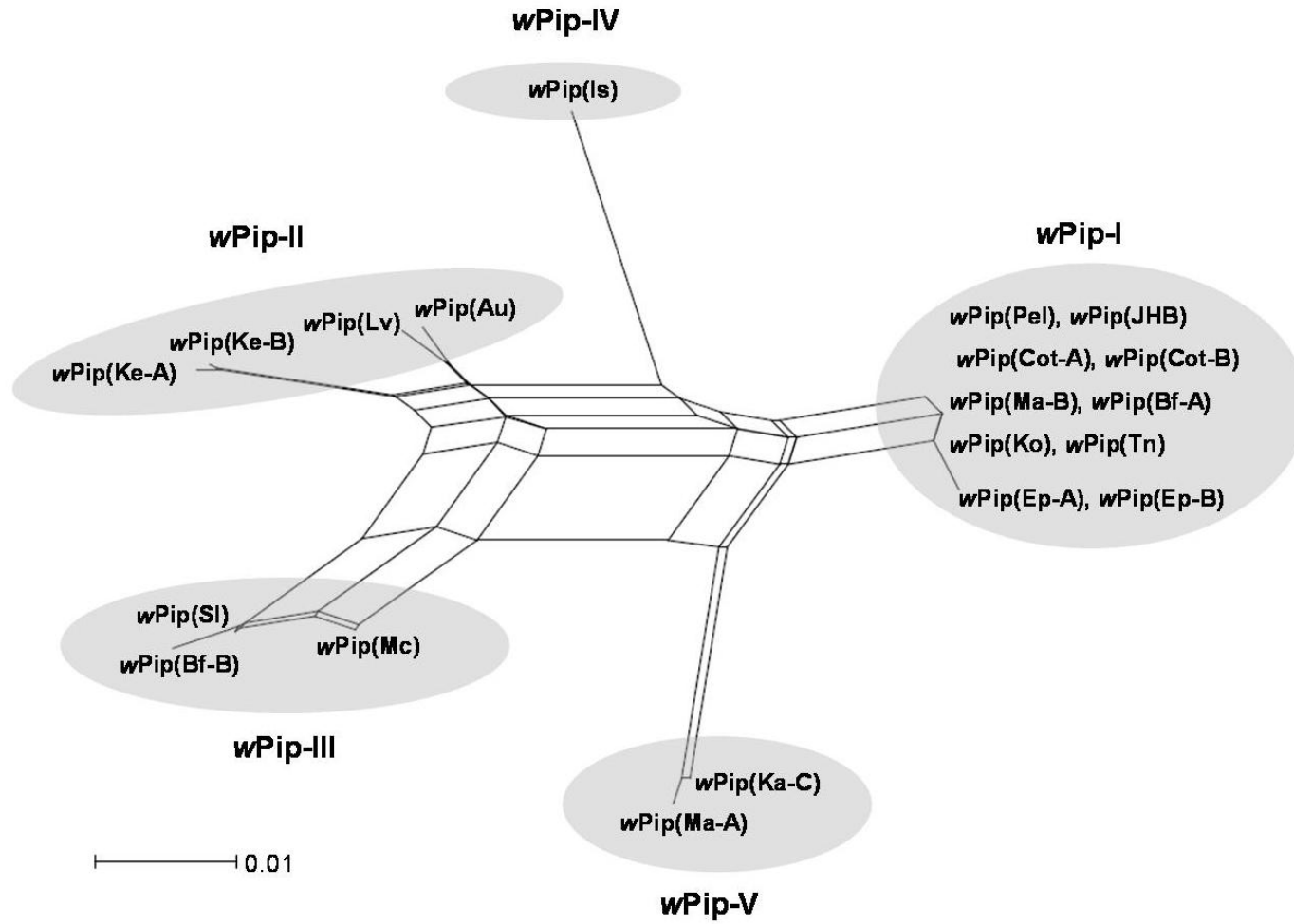


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Figure 2

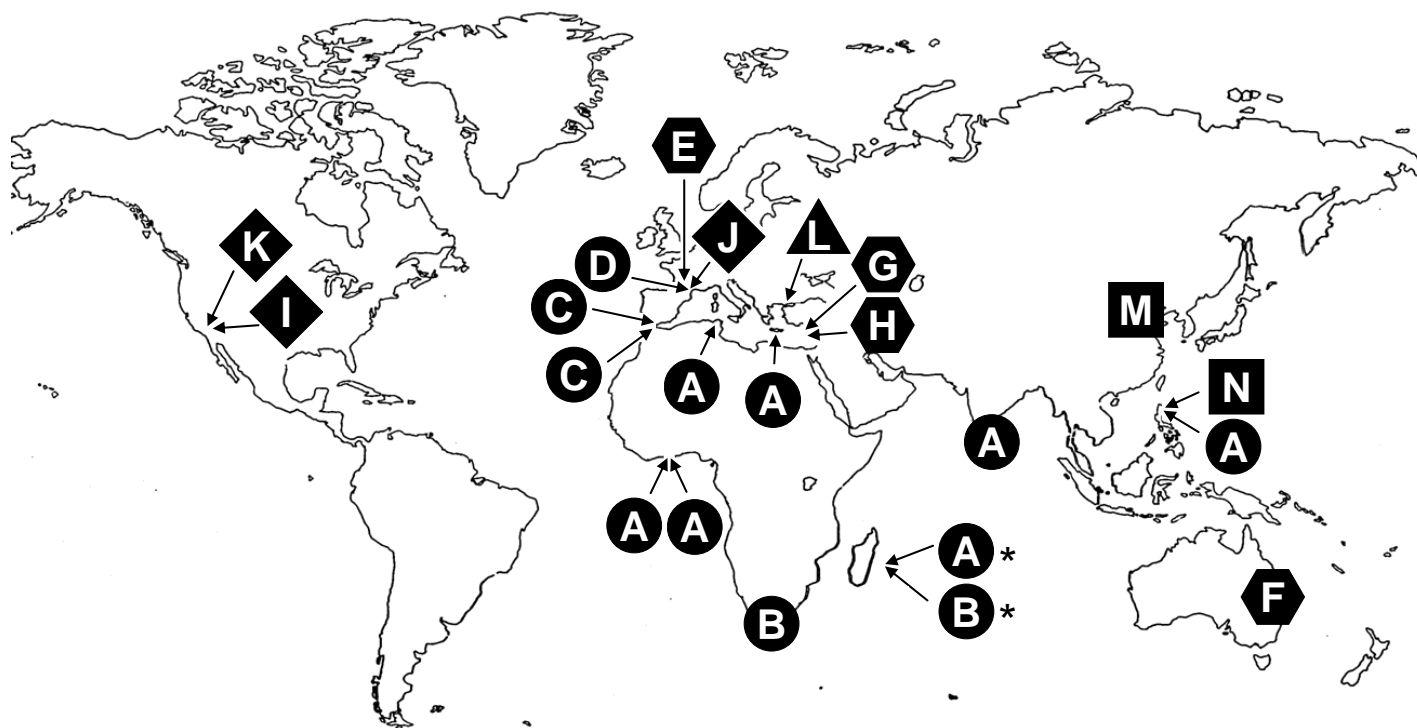


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Figure 3

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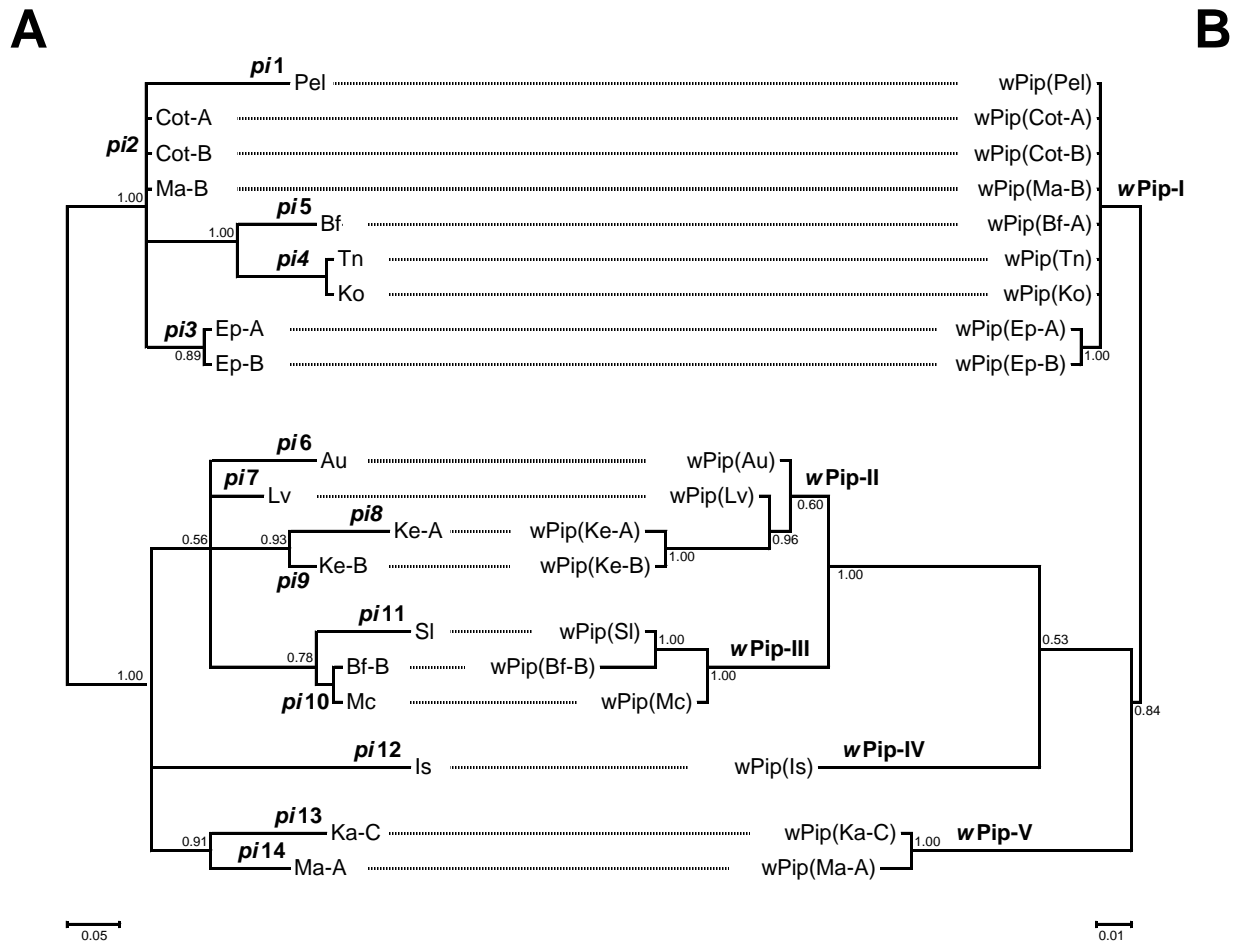
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Figure 4

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Figure 5