Genetic variation in target-site resistance to pyrethroids and pirimicarb in Tunisian populations of the peach potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

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Abstract

BACKGROUND: We used molecular assays to diagnose resistance to pyrethroids and pirimicarb in samples of *Myzus persicae* from field crops or an insect suction trap in Tunisia. Genotypes for resistance loci were related to ones for polymorphic microsatellite loci in order to investigate breeding systems, patterns of genetic diversity and to inform resistance management tactics.

RESULTS: The *kdr* mutation L1014F conferring pyrethroid resistance was found in all samples. The M918T s-*kdr* mutation also occurred in most samples, but only in conjunction with *kdr*. We discovered a previously unreported genotype heterozygous for L1014F but homozygous for M918T. Samples with modified acetylcholinesterase (MACE) conferring resistance to pirimicarb were less common but widespread. 16% of samples contained both the *kdr* and MACE mutations. Many unique microsatellite genotypes were found, suggesting that *M. persicae* is holocyclic in Tunisia. There were no consistent associations between resistance and microsatellite markers.

CONCLUSION: This first study of insecticide resistance in *M. persicae* in North Africa showed genetic variation in insecticide resistance within microsatellite multilocus genotypes (MLG_{MS}) and the same resistance mechanisms to be present in different MLG_{MS}. This contrasts with variation in northern Europe where *M. persicae* is fully anholocyclic. Implications for selection and control strategies are discussed.

Keywords: *Myzus persicae*; insecticide resistance; knockdown resistance; acetylcholinesterase; microsatellite polymorphism; holocycly; resistance management

1 INTRODUCTION

Aphids (Hemiptera: Aphididae) are major agricultural pests that cause extensive damage to crops through both direct feeding and disease transmission.[1,2] Insecticides are the main means of control, but insecticide resistance has been reported in approximately 20 aphid species[3] including the peach-potato or green peach aphid, *Myzus persicae* (Sulzer). This species has a cosmopolitan distribution, is highly polyphagous, and can vector many plant viruses.[4] Insecticide resistance in *M. persicae* now extends to most classes of insecticide, including organophosphates, carbamates and pyrethroids.[5] The mechanisms conferring the most potent resistance in bioassays are those resulting from amino-acid substitutions leading to conformational changes in insecticide target sites. Target-site resistance to the carbamate insecticide pirimicarb involves a modification to the enzyme acetylcholinesterase (so-called MACE resistance) and results from a serine to phenylalanine substitution (S431F) within the enzyme's active site.[6,7,8] Knockdown resistance (kdr) conferring resistance to pyrethroids involves mutations in the voltage-gated sodium channel protein in nerve membranes. The best documented substitutions are leucine to phenylalanine (L1014F, '*kdr*') and methionine to threonine (M918T, 's-*kdr*').[9,10] Another substitution at the 918 site (M918L) has recently been described.[11] In addition, resistance of *M. persicae* to neonicotinoids is attributable to enhanced activity of a P450 monooxygenase enzyme,[12] and/or a mutation (R81T) in the nicotinic acetylcholine receptor.[13] These mechanisms frequently coexist, greatly limiting the availability of effective control agents.[14]

Although primarily a consequence of insecticide use, the evolution of resistance in *M. persicae* is also influenced by its life-cycle and other aspects of the agroecosystem.[15,16,17,18] Alternation between sexual and asexual reproduction (holocycly) can favour resistance, since sexual reproduction and recombination leads to new genetic combinations on which selection for resistance can act, while multiple asexual generations can promote the rapid build-up of

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74 resistant individuals under exposure to insecticides. Both active flight and human transport of

75 individuals can contribute to the dispersal of resistance genotypes to new regions.

76 In southern Europe, *M. persicae* is holocyclic with one generation of sexual reproduction in

77 autumn on peach (*Prunus persica* L.), its primary host. Offspring from this sexual stage

78 disperse from peach to secondary host plants (herbaceous crops and weeds) and reproduce by

79 parthenogenesis during spring and summer.[4] However, in the absence of the primary host (as

80 in the UK), asexual reproduction on secondary host plants (anholocycly) persists throughout

81 the year.[15] In areas where holocycly predominates, *M. persicae* can be exposed to insecticides

82 on both primary and secondary host plants, enhancing the selection pressure for resistance.

83 However, fitness costs incurred by resistant aphids may lead to selection against resistance

84 when insecticide use is relaxed.[19,20] Thus, the frequency of resistance can potentially follow

85 cyclical dynamics corresponding to alternating periods of selection for resistance in spring

86 and summer and selection against resistance in autumn and winter.[14,21,22] Spatial and/or

87 temporal variation in host availability during different stages of the life-cycle can also affect

88 clonal dynamics and genetic diversity.[23,24,25] However, many aspects of resistance dynamics

89 and genetic diversity of *M. persicae* on its primary and secondary hosts remain poorly

90 understood.[26]

91 In Tunisia, *M. persicae* is a major vector of plant viruses to crops such as potato, tomato,

92 pepper and tobacco. Potatoes in particular are vulnerable to the non-persistent viruses *Potato*

93 *virus Y* (PVY) and *Potato leafroll virus* (PLRV).[27,28,29] For several decades, insecticides

94 including pyrethroids, organophosphates and carbamates have been used extensively against

95 *M. persicae* and other aphid pests.[30] Repeated use of insecticides on potatoes and other crops

96 containing aphids imposes a continual risk of resistance developing. To date, however, there

97 has been no work on the status and dynamics of insecticide resistance in *M. persicae* in North

98 Africa. We report here on the use of established molecular assays to diagnose the status of

99   resistance to pyrethroids and pirimicarb in samples of *M. persicae* collected from field crops

100   and from an insect suction trap in northern Tunisia, where crops vulnerable to attack by this

101   aphid are concentrated. Resistance profiles disclosed by these assays are then compared the

102   diversity of genotypes at five polymorphic microsatellite loci in order to investigate the

103   genetic composition and clonal diversity of *M. persicae* and inform attempts at resistance

104   management.

105   **2 MATERIALS AND METHODS**

106   **2.1 Aphid samples**

107   Samples of *M. persicae* were collected from peach (*Prunus persica*) orchards and potatoes

108   (*Solanum tuberosum*) at four sites in Tunisia (Fig. 1): (i) **Cap bon** (36°40'40"N 10°28'20"E)

109   a coastal sub-humid zone with Kermes oak (*Quercus* cf. *coccifera* L.) forest, *Cystus* sp. and

110   various crops (fruit trees, potato, tomato, pepper); (ii) **Manouba** (36°43'09"N 9°29'10"E) a

111   semi-arid area with warm winter zone characterized by Oleo-lentisc forest with mixed

112   farming; (iii) **Jendouba** (36°33'42"N 8°56'40"E) a continental sub-humid zone with Oleo-

113   lentisc forest and cereal crops, sugar beet and vegetables; (iv) **Kairouan** (35°39'50"N

114   9°59'10"E), a continental zone with arid cold winter and steppe with a developing agricultural

115   industry based on fruit trees and vegetables. Kairouan is a new site for producing Spunta and

116   Nicola seed potatoes. Collections were made during five consecutive years. In the majority of

117   cases, samples were taken twice per year in the spring and autumn, enabling a comparison of

118   insects before and after the growing (=crop protection) season. At each site, aphids were

119   collected from widely-spaced plants in order to limit the chance of sampling the same colony.

120   In addition, aphids were obtained from a 12.2m suction trap at Cap bon and these were

121   shipped in alcohol. Aphids confirmed as *M. persicae* were stored in microtubes filled with

122   70% ethanol and preserved at -80 C prior to genotypic testing. In total, 32 samples (26 from

123   field sites and 6 from suction traps), totalling 903 individuals were obtained. Sampling sites,

124   host plants and dates of collection are listed in table 1.

125   **2.2 DNA extraction**

126   Total genomic DNA was extracted from individual adult aphids using DNAzol (Invitrogen,

127   Carlsbad, California) at one fifth scale of the supplier's recommended protocol.

128   (http://www.invitrogen.com/content/sfs/manuals/10503.pdf). Each aphid was dried in a speed-

129   vac and then crushed using a Teflon pestle in a microcentrifuge tube in 200 μl of DNAzol

130   containing 1% (v/v) of polyacryl carrier (Invitrogen, Carlsbad, California). The homogenate

131   was centrifuged for 12 min at 10,000g after a 30 min incubation period at room temperature.

132   The supernatant was transferred to a new tube and a half volume of 100% ethanol was added.

133   The tube was cooled to - 20°C for 30 min and DNA pelleted by centrifuging at 10,000g for 15

134   min. The DNA pellet was dissolved in 50 μl of distilled, deionized water (ddH2O) after being

135   washed twice with 70% ethanol. The quality and quantity of DNA samples were assessed by

136   spectrophotometry (Nanodrop Technologies) and by running an aliquot on a 1% agarose gel.

137   All DNA samples were diluted to 40 ng/μl and stored at -20°C for future use.

138   **2.3 Insecticide resistance mechanisms**

139   Mutations conferring knockdown and MACE resistance were identified using TaqMan assays

140   to discriminate between wildtype and resistance alleles.[31,32] Reactions took place in a

141   STRATAGENE MX 3000 (Agilent Technologies, Santa Clara, CA) thermocycler. Diagnosis

142   of the presence or absence of the *kdr* (L1014F) and s-*kdr* (M918T) mutations in the sodium

143   channel gene, and of MACE (S431F) in the acetylcholinesterase gene, enabled each

144   individual to be classified as homozygous susceptible (SS), heterozygous (SR) or

145   homozygous resistant (RR) at each of the three loci. This in turn enabled each individual to be

146   allocated a multi-locus resistance genotype (MLG$_R$).

147   **2.4 Microsatellite genotyping**

148　A sub-sample of 153 individuals was subjected to microsatellite analysis to investigate the

149　genotypic variation of aphids from peach and potato at three of the collection sites. Each

150　aphid was genotyped using five microsatellite loci M40, M49, M63, M86 and myz9[33,34]

151　chosen on the basis of their level of pulymorphism (allele numbers of 7, 15, 11, 11 and 11,

152　respectively). These loci were amplified using fluorochrome primers labelled at the 5'end of

153　the reverse primer (M40 FAM, M49 HEX, M63 FAM, M86 TET, myz9 HEX; MWG

154　Biotech, Germany) and PCR ready-to-go beads (Amersham Biosciences, U.K.; for the

155　conditions used, see[35]). Products were then analysed on an ABI 377 (96) automated

156　sequencer with Genescan v3.4 and Genotyper v2.5 software (Applied Biosystems, Foster

157　City, California), for both visualization and analyses. Each individual was described by its

158　multilocus microsatellite genotype (MLG_M): the combination of alleles at all five

159　microsatellite loci.

160　**2.3 Statistical analysis**

161　*2.3.1 Genetic variability within samples*

162　Allele frequencies, mean number of alleles per locus and allelic richness were calculated

163　using FSTAT version 2.9.3.2.[36] Linkage disequilibrium (LD) between loci within each

164　population and departure from H-W equilibrium at each locus were tested using ARLEQUIN

165　version 3.11.[37]

166　*2.3.2 Genetic variation between samples*

167　Samples were pooled in three ways: (i) by geographical origin, (ii) by host plant, and (iii) by

168　year of collection. Population structure was assessed by calculating multilocus F_{ST} values[38]

169　for pairwise comparisons of samples using ARLEQUIN version 3.11.[37] The null distribution

170　of pairwise F_{ST} values under the hypothesis of no difference between the populations is

171　obtained by permuting diploid multilocus genotypes between populations. The *P* value of the

172　test is the proportion of 100 000 permutations leading to an F_{ST} value larger than or equal to

173   the observed one. The structure of the data was also investigated by analysis of molecular

174   variance[39] (AMOVA) using Arlequin version 3.11. A permutation non-parametric approach

175   was used for the significance of fixation indices described in Excoffier *et al.*[39]. Allelic

176   differentiation between populations was examined using GENEPOP version 3.4. An unbiased

177   estimate of the *P* value of the Fisher exact test was made using a Markov chain method

178   described in Raymond and Rousset.[40] For microsatellite markers, analyses were performed

179   without clonal copies, i.e., with the data reduced to a single representative of each multilocus

180   genotype (MLG_M) per population, because the clonal amplification of genotypes inevitably

181   leads to deviations from genetic equilibria.[41,42]

182   **3 RESULTS**

183   **3.1 Frequency of insecticide resistance genes**

184   *3.1.1 Kdr resistance*

185   The *kdr* mutation L1014F was present in heterozygous or homozygous form in 65% of

186   individuals collected from the field crops (Table 1). The frequency of genotypes containing

187   *kdr* varied between 3 and 100% for samples from both peach and potato across all years, with

188   heterozygotes (RS) being the most common resistance genotype. This mutation was most

189   frequent at Cap bon and Kairouan, and least frequent at Jendouba. The s-*kdr* mutation M918T

190   was present in 21.7% of individuals collected from the field crops and was only ever found in

191   conjunction with *kdr* (ie. no SSRS or SSRR genotypes; Table 2). This mutation was also

192   widespread, being absent in only four of the 26 samples analysed (Table 1) and most frequent

193   at the Kairouan locality. The resistance genotypes characterised included one heterozygous

194   for L1014F but homozygous for M918T (SRRR) that has never been reported previously. The

195   three most common knockdown resistance genotypes were SSSS (neither resistance

196   mutation), SRSS (heterozygous for *kdr*, homozygous susceptible for s-*kdr*) and SRRR

197   (heterozygous for *kdr*, homozygous resistant for s-*kdr*) (Table 2).With all data collated, *kdr*

198 and s-*kdr* mutations were in very strong linkage disequilibrium (P < 0.001), as expected

199 because of the close positioning of these two sites in the same gene.

200 *3.1.2 MACE resistance*

201 23.6% of the individuals collected from field crops were either heterozygous or homozygous

202 for the S431F mutation (Table 1), which was found at all four collection sites. It was most

203 common at Manouba, with heterozygotes being the most frequent genotype. Moreover, 16.4%

204 of individuals possessed both the *kdr* and MACE mutations, and 5.3% had all three resistance

205 mutations (*kdr,* s-*kdr* and MACE). There was no significant linkage disequilibrium between

206 MACE and *kdr* or MACE and s-*kdr* using data pooled across all collections (*P*=0.08 and 0.97,

207 respectively).

208 **3.2 Differences in resistance genotypes between crops and locations**

209 The *kdr* mutation was significantly more frequent (Fisher's exact test $P < 10^{-5}$) in aphids from

210 peach (73.3%) than in those from potato (31.7%). The F_{ST} values revealed significant

211 variation in MLG_R frequencies between locations (Table 3). There was no significant

212 difference between years in the same locality, thereby justifying the pooling of samples across

213 years for each locality. Pairwise comparisons between locations all yielded highly significant

214 levels of differentiation. A hierarchical AMOVA also revealed a significant differentiation

215 (F_{ST}=0.101; P < 0.001) over all mechanisms and locations, with most of the variance (89.8%)

216 being within location. The *kdr* mutation explained a high percentage of the between-locality

217 variance (12.17%) with an F_{ST} value of 0.121 (P < 0.001).

218 **3.3 Suction trap samples and their relationship to field samples**

219 Compared to collections from field crops, which are more likely to reflect localized events

220 including insecticide treatment regimes, collections from suction traps should be

221 representative of larger areas.[43] Our analysis was limited to comparisons between samples of

222 *M. persicae* collected from the field and a suction trap at a single site (Cap bon) (Table 1 and

223 4), due to the absence of suction traps at another sites. Each of the three resistance mutations

224 (*kdr*, s-*kdr* and MACE) had a similar prevalence in samples collected from field crops and

225 from the suction trap at Cap bon. For example, the *kdr* mutation L1014F was present in 65%

226 of individuals collected from field crops and 61.5% of individuals collected from the suction

227 trap. Similarly, the S431F mutation was detected in 23.6% of individuals collected from field

228 crops and in 22.7% of individuals collected from the suction trap.

229 **3.4 Microsatellite genotypes**

230 The number of alleles identified for each microsatellite locus ranged from eight at M40 and

231 M63 to 15 at M49. 49 alleles were detected across all loci. All 13 samples used for

232 microsatellite analysis showed substantial genetic diversity, as expressed by the mean number

233 of alleles per locus and the randomization test for population allelic richness (Table 5). Values

234 of allelic richness ranged from 1.9 at locus M40 to 5.0 at locus M49.

235 120 different MLG_{MS} were found among 153 individuals genotyped. Estimates of clonal

236 diversity (G) measured by dividing the numbers of genotypes by the number of individuals,[16]

237 ranged from 0.62 to 1 across the 13 samples (Table 5). Only 16 of the 120 MLG_{MS} were

238 found more than once (between two and six times in the samples). Most genotypes were

239 unique to a particular sampling site although one was collected at multiple sites. When

240 considering only one individual per MLG_M, significant linkage disequilibrium was detected

241 only for a single pair of loci (M40 $\times$ M63, *P*<0.001). No linkage disequilibrium was detected

242 between microsatellite loci and mutations conferring insecticide resistance with the exception

243 of a significant association between s-*kdr* and M40.

244 **3.5 Comparing MLG_{MS} between samples**

245 Comparisons of genetic differentiation for microsatellite loci between locations or between

246 host plants showed significant differentiation between the three locations (Table 6). A

247 hierarchical AMOVA also revealed a significant F_{ST} (0.040; *P* < 0.001) variation over all loci

248 and locations, with most of the variance (94%) being within location. There was low but

249 significant genetic differentiation between peach and potato samples (P< 0.001; F_{ST} = 0.024).

250 **3.6 Comparison of resistance and microsatellite profiles**

251 Among the 120 different MLG_Ms identified using microsatellite markers, only seven

252 contained more than one MLG_R defined using the three resistance markers. Of the remaining

253 MLG_Ms, 18 were susceptible for both resistance mechanisms, 95 contained the *kdr* mutation,

254 31 the s-*kdr* mutation and 28 the MACE mutation. Only 16 MLG_Ms contained both the *kdr*

255 and MACE mutations. No strict associations could be established between microsatellite and

256 resistance profiles since: (a) with one exception (see above) no significant linkage could be

257 detected between microsatellites and insecticide resistance markers; (b) each resistance

258 mechanism was found in different MLG_Ms; and (c) some resistance genes showed variation

259 within MLG_Ms.

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261 **4 DISCUSSION**

262 Heterozygotes and homozygotes for mutations conferring resistance to pyrethroids and

263 pirimicarb were readily found using allelic discrimination PCR assays[31,32], showing that

264 resistance to these functionally distinct compounds is well established in Tunisia. Differences

265 between samples in the frequency of resistance mechanisms may reflect some spatial

266 variation in selection pressure. The *kdr* (L1014F) mutation was present at all four collection

267 sites and in samples from the single suction trap, in some cases in 100% of the insects tested.

268 The frequencies of *kdr* were statistically higher in peach orchards than in potato fields,

269 possibly a consequence of the selection pressure imposed by spring treatments in peach

270 orchards against *M. persicae* and other pests including Mediterranean fruit fly *Ceratitis*

271 *capitata*, Peach Twig Borer *Anarsia lineatella* and scale insects. Both the *kdr* and s-*kdr*

272 mutations have now been identified in samples of *M. persicae* worldwide, and the status of

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273   knockdown resistance in Tunisia mirrors its generally high frequency in Europe, USA and

274   Japan[44,14,25] where it is a major constraint on the continuing use of pyrethroids for combating

275   *M. persicae.*

276   Only some of the possible genotypic combinations of the *kdr* and s-*kdr* mutations were

277   detected. Three alleles predominated: fully susceptible (SSSS), heterozygous at *kdr* but

278   homozygous susceptible at s-*kdr* (SRSS), and heterozygous at *kdr* but homozygous resistant

279   at s-*kdr* (SRRR). Prior to this study, M918T had never been observed in the absence of

280   L1014F, leading to an assumption that both mutations are necessary for an enhanced

281   resistance phenotype. The presence of the previously unreported SRRR genotype in our

282   samples demonstrates the occurrence of an allele that is wild-type at the *kdr* locus but which

283   contains the s-*kdr* mutation. The phenotype of insects with this genotype in terms of the

284   expression and potency of resistance has not been investigated. The existence of the new

285   allele implies that aphids with a SSRR genotype should be generated through outcrossing,

286   although none were detected in the samples investigated.

287   No individuals were found that were homozygous resistant for both mutations (RRRR).

288   Fenton *et al.*[15] also found a lack of the RRRR genotype in Scotland. The lack of such double

289   homozygotes could implicate a fitness cost associated with such a genotype.[45,46] The absence

290   of homozygous genotypes for *kdr* and s-*kdr* also matches observations in populations of *M.*

291   *persicae* in mainland Europe, Zimbabwe and South East Australia, where there appears to be

292   a strong selection pressure against homozygosity in *kdr* due to the high fitness costs

293   associated with the trait.[46]

294   The frequency of MACE resistance was generally constant between years. Its relatively

295   limited frequency in Tunisia could be due to a switch to insecticides other than pirimicarb,

296   resulting in a situation where MACE is of no advantage. Although, as expected, there was

297   strong linkage disequilibrium between *kdr* and s-*kdr*, there was no significant association

298   between MACE and either the *kdr* or s-*kdr* mutations, which could arise in areas treated with

299   both pyrethroids and pirimicarb. In Tunisia, such an association is not apparent, presumably

300   as a consequence of recombination during sexual reproduction.

301   The presence of 120 microsatellite genotypes in 153 individuals indicates a high level of

302   genetic diversity similar to that found in *M. persicae* in France, where 100 genotypes were

303   identified from 174 aphids collected from suction traps in 2000.[25] This level of variation

304   contrasts markedly with that in Scotland (UK), where only 21 different genotypes were found

305   in 1497 individuals collected from suction traps and secondary hosts.[18] This lack of variation

306   was attributed to obligate anholocycly, since the primary host is absent. In Greece, the extent

307   of variation in microsatellite markers was closely associated with the presence or absence of

308   the primary host, with the number of unique MLG_{MS} being much higher in peach-growing

309   areas than in non-peach-growing areas[4]. Thus, the absence of genetic signatures of clonal

310   reproduction (repeated genotypes, linkage disequilibrium) suggests that Tunisian samples are

311   mostly constituted of cyclically parthenogenetic aphids. The variation observed was

312   attributable to the fact that most of our collections were made from peach trees in spring, and

313   were offspring of the founding females that emerged from sexually-produced eggs.

314   Significant pairwise F_{ST} values for pooled samples from different localities imply genetic

315   differentiation even over small distances (Cap bon and Kairouan are less than 150 km apart,

316   Fig. 1). For *Sitobion avenae*, Simon *et al.*[47] obtained an average F_{ST} value of 0.032 in France,

317   and Llewellyn *et al.*[48] reported most values lower than 0.05 in the UK. For *Rhopalosiphum*

318   *padi*, Delmotte *et al.*[49] reported values of 0.022 and 0.032 for anholocyclic and cyclically

319   parthenogenetic genotypes, respectively. The authors suggested that genetic homogeneity

320   over a large geographical scale results from the high migratory habits of two aphid pests of

321   cereal crops. However, *M. persicae* seems to differ in this respect, as shown also by previous

322   studies in Australia[24] (F_{ST} = 0.058–0.202, with an average 0.087); France[25] (F_{ST} up to 0.17–

323 0.21 in some cases) and Greece[50] (F_{ST} = 0.05–0.174, with an average 0.062). It should be

324 noted that local differentiation does not mean that intense migration and long-distance flights

325 do not occur in *M. persicae*, but rather that long-distance migration may be rare or that the

326 success rate of migration may be low.[51] Tunisian samples showed variation in insecticide

327 resistance genotypes (MLG$_{RS}$) was present within MLG$_{MS}$ and that the same insecticide

328 resistance mutations were present in different MLG$_{MS}$. This again points to the existence of

329 sexual recombination. Recombination resulting from sexual reproduction can lead to a

330 polymorphism at resistance genes within MLGs, and this conclusion is supported by the

331 presence of peach, the primary host, and by the high level of genetic diversity revealed by

332 microsatellite analysis.

333 In conclusion, molecular analyses of the diversity of insecticide resistance mutations

334 in *M. persicae* can assist in determining the levels and types of resistance mechanism present.

335 This information can strengthen strategies for preserving the effectiveness and increasing the

336 performance of insecticides currently used for managing *M. persicae*, and for deploying

337 insecticides alongside other control strategies. Work in cropping systems in Tunisia has

338 helped to reveal how patterns of variation in insecticide resistance genes relate to those in

339 microsatellite markers and compliments information from other continents to provide a global

340 perspective on the evolution of resistance in one of the world's most economically-significant

341 agricultural pests.

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365   **REFERENCES**

366   1. Blackman RL and Eastop VF, Aphids on the world's crops, an identification and

367   information guide. Chichester, UK: John Wiley & Sons Ltd (2000).

368   2.   Moury B, Fabre F and Senoussi R, Estimation of the number of virus particles transmitted

369   by an insect vector. *Proc Natl Acad Sci U S A* **104**:17891–17896 (2007).

370   3.   van Emden H and Harrington R, Aphids as Crop Pests. CABI North American Office,

371   Cambridge, Massachusetts (2007).

372   4.   Blackman RL, Malarky G, Margaritopoulos JT and Tsitsipis JA, Distribution of common

373   genotypes of *Myzus persicae* in Greece, in relation to life cycle and host plant. *Bull*

374   *Entomol Res* **97**: 253–263 (2007).

375   5.   Bass C, Puinean M, Zimmer CT, Denholm I, Field LM, Foster SP *et al.*, The evolution of

376   insecticide resistance in the peach-potato aphid, *Myzus persicae*. *Insect Biochem Mol Biol*

377   **51**:41–51 (2014).

378   6.   Moores GD, Devine GJ and Devonshire AL, Insecticide insensitive acetylcholinesterase

379   can enhance esterase-based resistance in *Myzus persicae* and *Myzus nicotianae*, *Pestic*

380   *Biochem Physio* **49**:114–120 (1994).

381   7.   Andrews MC, Williamson MS, Callaghan A, Field LM and Moores GD, A single amino

382   acid substitution found in pirimicarb-insensitive acetylcholinesterase of the peach potato

383   aphid, *Myzus persicae* (Sulzer). Proceedings of the XI International Symposium on

384   Cholinergic Mechanisms: Function and Dysfunction. St Moritz, Switzerland, p. 38 (2002).

385   8.   Nabeshima T, Kozak T, Tomita T and Kono Y, An amino acid substitution on the second

386   acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, *Myzus*

387   *persicae*. *Biochem Biophys Res Commun* **307**:15–22 (2003).

388   9.   Martinez-Torres D, Foster SP, Field LM, Devonshire AL and Williamson MS, A sodium

389   channel point mutation is associated with resistance to DDT and pyrethroid insecticides in

390   the peach–potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Insect Mol Biol*

391   **8**:339–346 (1999).

392   10. Eleftherianos I, Foster S, Goodson S, Williamson M and Denholm I, Toxicological and

393   molecular characterization of pyrethroid knockdown resistance (kdr) in the peach-potato

394   aphid, *Myzus persicae* (Sulzer) Aphids in a new millennium. Proceedings of the Sixth

395   International Symposium on Aphids, September 2001, Rennes, France, pp. 213–218

396   (2004).

397   11. Fontaine S, Caddoux L, Brazier C, Bertho C, Bertolla P, Micoud A and Roy L,

398   Uncommon associations in target resistance among French populations of *Myzus persicae*

399   from oilseed rape crops. *Pest Manag Sci* **67**:881–885 (1999).

400   12. Puinean AM, Foster SP, Oliphant L, Denholm I, Field M, Millar NS *et al.*, Amplification

401   of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in

402   the aphid *Myzus persicae*. *Plos Genet* **6**: e1000999 (2010).

403   13. Bass C, Puinean AM, Andrews MC, Culter P, Daniels M, Elias J *et al.*, Mutation of a

404   nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid

405   insecticides in the aphid *Myzus persicae*. *BMC Neurosci* **12**:51 (2011).

406   14. Foster SP, Denholm I. and Devonshire AL, The ups and downs of insecticide resistance in

407   peach-potato aphids (*Myzus persicae*) in the UK. *Crop Prot* **19**:873–879 (2000).

408   15. Fenton B, Malloch G, Woodford JAT, Foster SP, Anstead J, Denholm I *et al.*, The attack

409   of the clones: tracking the movement of insecticide-resistant peach–potato aphids *Myzus*

410   *persicae* (Hemiptera: Aphididae). *Bull Entomol Res* **95**:483–494 (2005).

411   16. Zamoum T, Simon JC, Crochard D, Ballanger Y, Lapchin L, Vanlerberghe-Masutti, F.

412   and Guillemaud T, Does insecticide resistance alone account for the low genetic

413   variability of asexually reproducing populations of the peach–potato aphid *Myzus*

414   *persicae*? *Heredity* **94**:630–639 (2005).

415   17. Margaritopoulos JT, Skouras PJ, Nikolaidou P, Manolikaki J, Maritsa K, Tsamantani K *et al.*, Insecticide resistance status of *Myzus persicae* (Hemiptera, Aphididae) populations from peach and tobacco in mainland Greece, *Pest Manag Sci* **63**:821–829 (2007).

418   18. Kasprowicz L, Malloch GL, Pickup J and Fenton B, Spatial and temporal dynamics of Myzus persicae clones in fields and suction traps. *Agr Forest Entomol* **10:**91–100 (2008).

420   19. Foster SP, Harrington R, Devonshire AL, Denholm I, Clark SJ and Mugglestone MA, Evidence for a possible fitness trade-off between insecticide resistance and the low temperature movement that is essential for survival of UK populations of *Myzus persicae* (Hemiptera: Aphididae). *Bull Entomol Res* **87**:573–579 (1997).

424   20. Foster SP, Denholm I, Poppy GM and Thompson RW, Powell Fitness trade-off in peach-potato aphids (*Myzus persicae*) between insecticide resistance and vulnerability to parasitoid attack at several spatial scales. *Bull Entomol Res* **101**:659–666 (2011).

427   21. Foster SP, Denholm I and Devonshire AL, Field–simulator studies of insecticide resistance to dimethylcarbamates and pyrethroids conferred by metabolic-and target site-ased mechanisms in peach–potato aphids, *Myzus persicae* (Hemiptera: Aphididae). *Pest Manag Sci* **58**:811–816 (2002).

431   22. Fenton B, Margaritopoulos JT, Malloch, GL and Foster SP, Micro-evolutionary change in relation to insecticide resistance in the peach-potato aphid, *Myzus persicae*. *Ecol Entomol* **35**:131–146 (2010).

434   23. Vorburger C, Temporal dynamics of genotypic diversity reveal strong clonal selection in the aphid *Myzus persicae*. *J Evol Biol* **19**:97–107 (2006).

436   24. Wilson ACC, Sunnucks P, Blackman RL and Hales DF, Microsatellite variation in cyclically parthenogenetic populations of *Myzus persicae* in south-eastern Australia. *Heredity* **88**:258–266 (2002).

439 25. Guillemaud T, Brun A, Anthony N, Sauge MH, Boll R, Delorme R *et al.*, Incidence of
440   insecticide resistance alleles in sexually-reproducing populations of the peach-potato
441   aphid *Myzus persicae* (Hemiptera: Aphididae) from southern France. *Bull Entomol Res*
442   **93**:289–297 (2003).

443 26. Van Toor RF, Malloch GL, Anderson EA, Dawson G and Fenton B, Insecticide resistance
444   profiles can be misleading in predicting the survival of *Myzus persicae* genotypes on
445   potato crops following the application of different insecticide classes. *Pest Manag Sci*
446   **69**:93–103 (2013).

447 27. Djilani KF, Guyader S, Gorsane F, Khamassy N, Rouzé J, Marrakchi M and Fakhfakh H,
448   Diagnosis and molecular analysis of *Potato leafroll virus* isolates in Tunisia. *Bulletin*
449   *OEPP/EPPO* Bulletin **33**:361–368 (2003).

450 28. Boukhris BS, Hullé M, Rouzé-Jouan J, Glais L and Kerlan C, *Solanum eleagnifolium*, a
451   potential source of Potato virus Y (PVY) propagation. *Bulletin OEPP/EPPO* Bulletin
452   **37**:125–128 (2007).

453 29. Ben Khalifa M, Simon V, Marrakchi M, Fakhfakh H and Moury B, Contribution of host
454   plant resistance and geographic distance to the structure of Potato virus Y (PVY)
455   populations in pepper in Northern Tunisia. *Plant Pathol* **58**:763–772 (2009).

456 30. Raboudi F, Fattouch S, Makni H and Makni M, Biochemical and molecular analysis of
457   the pirimicarb effect on acetylcholinesterase resistance in Tunisian populations of potato
458   aphid Macrosiphum euphorbiae (Hemiptera: Aphididae) *Pest Biochem Physiol* **104**: 261–
459   266 (2012).

460 31. Anstead JA, Williamson MS, Eleftherianos IG and Denholm I, High-throughput detection
461   of knockdown resistance in *Myzus persicae* using allelic discriminating quantitative PCR
462   *Insect Biochem. Mol Biol* **34**:871–877(2004).

463 32. Anstead JA, Williamson MS and Denholm I, New methods for the detection of insecticide

464   resistant *Myzus persicae* in the U.K. suction trap network. *Agr Forest Entomol* **10**:291–

465   295 (2008).

466 33. Wilson ACC, Massonnet B, Simon JC, Prunier-Leterme N, Dolatti L, Llewellyn KS *et al.*,

467   Cross-species amplification of microsatellite loci in aphids: assessment and application.

468   *Mol Ecol Notes* **4**:104–109 (2004).

469 34. Sloane MA, Sunnucks P, Wilson ACC and Hales DF, Microsatellite isolation, linkage

470   group identification and determination of recombination frequency in the peach-potato

471   aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Genetic Res Cambridge* **77**: 251–

472   260 (2001).

473 35. Malloch G, Fenton B and Butcher RDJ, Molecular evidence for multiple infections of a

474   new subgroup of *Wolbachia* in the European raspberry beetle *Byturus tomentosus*. *Mol*

475   *Ecol* **9**:77–90 (2000).

476 36. Goudet J, Fstat, a program to estimate and test gene diversities and fixation indices

477   (version 2.9.3). Available from http:// www.unil.ch/izea/softwares/fstat.html. Updated

478   from Goudet (1995) (2001).

479 37. Excoffier L, Laval G and Schneider S, ARLEQUIN: An Integrated Sofware Package for

480   Population Genetics Data Analysis. CMPG, Bern, Swissland (2005).

481 38. Weir BS and Cockerham CC, Estimating F-statistics for the analysis of population

482   structure. *Evolution* **38**:1358–1370 (1984).

483 39. Excoffier L, Smouse PE and Quattro JM, Analysis of molecular variance inferred from

484   metric distances among DNA haplotypes: application to human mitochondrial DNA

485   restriction data. *Genetics* **131**:479–491 (1992).

486 40. Raymond M and Rousset F, Genepop (version. 1.2) A population genetics software for

487   exact tests and ecumenicism. *J Heredity* **86**:248–249 (1995).

488   41. Sunnucks P, Barro PJd, Lushai G, Maclean N and Hales D, Genetic structure of an aphid

489   studied using microsatellites: cyclic parthenogenesis, differentiated lineages and host

490   specialization. *Mol Ecol* **6**:1059–1073 (1997).

491   42. Halkett F, Plantegenest M, Prunier-Leterme N, Mieuzet L, Delmotte F and Simon JC,

492   Admixed sexual and facultatively asexual aphid lineages at mating sites. *Mol Ecol* **14**:

493   325–336 (2005).

494   43. Taylor LR, In Movement of highly mobile insects: Concepts and methodology in research

495   (Eds, R. L. Rabb and G. G. Kennedy) *University Graphics, Raleigh, North Carolina*, 148–

496   185 (1979).

497   44. Field LM, Anderson AP, Denholm I, Foster SP, Harling ZK, Javed N *et al.*, Use of

498   biochemical and DNA diagnostics for characterising multiple mechanisms of insecticide

499   resistance in the peach-potato aphid, *Myzus persicae* (Sulzer). *Pestic Sc* **51**:283–289

500   (1997).

501   45. Foster SP, Young S, Williamson MS, Duce I, Denholm I and Devine GJ, Analogous

502   pleiotropic effects of insecticide resistance genotypes in peach-potato aphids and

503   houseflies. *Heredity* **91**:98–106 (2003).

504   46. Anstead JA, Mallet J and Denholm I, Temporal and spatial incidence of alleles conferring

505   knockdown resistance to pyrethroids in the peach-potato aphid, *Myzus persicae*

506   (Hemiptera: Aphididae), and their association with other insecticide resistance

507   mechanisms. *Bull Entomol Res* **97**:243–252 (2007).

508   47. Simon JC, Baumann S, Sunnucks P, Hebert PDN, Pierre JC, Le Gallic JF and Dedryver

509   CA, Reproductive mode and population genetic structure of the cereal aphid *Sitobion*

510   *avenae* studied using phenotypic and microsatellite markers. *Mol Ecol* **8**:531–545 (1999).

511   48. Llewellyn KS, Loxdale HD, Harrington R, Brookes CP, Clark SJ and Sunnucks P,

512   Migration and genetic structure of the grain aphid (*Sitobion avenae*) in Britain related to

climate and clonal fluctuation as revealed using microsatellites. *Mol Ecol* **12**:21−34 (2003).

49. Delmotte F, Leterme N, Gauthier JP Rispe C and Simon JC, Genetic architecture of sexual and asexual populations of the aphid *Rhopalosiphum padi* based on allozyme and microsatellite markers. *Mol Ecol* **11**:711−723 (2002).

50. Margaritopoulos JT, Malarky G, Tsitsipis JA and Blackman RL, Microsatellite DNA and behavioural studies provide evidence of host-mediated speciation in *Myzus persicae* (Hemiptera: Aphididae). *Biological Journal of the Linnean Society* **91**:687−702 (2007).

51. Vorburger C, Lancaster M and Sunnucks P, Environmentally related patterns of reproductive modes in the aphid *Myzus persicae* and the predominance of two 'superclones' in Victoria, Australia. *Mol Ecol* **12**:3493−3504 (2003).

525   **Figure caption**

526   Figure 1. Map of Tunisia showing the location of sampling sites.

527

Table 1. Percentage of *M. persicae* individuals with different resistance genotypes, collected

from field crops in Tunisia.

Location	Crop	Date	Abbreviation*	n	*Kdr* genotype			super-*kdr* genotype			MACE genotype		
					SS	*SR*	*RR*	*SS*	SR	RR	SS	SR	RR
Cap bon	Peach	20/05/2005	Cpe5	30	16.6	63.3	20.0	76.7	0.0	23.3	76.7	16.7	6.66
	Peach	24/03/2006	Cpe6	77	14.3	55.8	29.9	77.9	1.29	20.8	85.7	7.79	6.49
	Peach	01/12/2006		20	20.0	75.0	5.0	90.0	5.0	5.0	70.0	30.0	0.0
	Peach	27/04/2007		21	38.1	19.0	42.9	81.0	0	19.0	33.3	952	57.1
	Peach	18/04/2008	CpeL8	43	23.3	62.8	13.9	76.7	9.30	13.9	79.1	18.6	2.32
	Peach	26/10/2008	CpeE8	34	2.92	91.2	5.88	73.5	2.84	23.6	61.8	35.3	2.94
	Peach	23/04/2009	Cpe9	56	8.92	83.9	7.14	66.1	3.57	30.4	69.6	21.4	8.92
	Potato	21/04/2006	Cpt6	20	85.0	15.0	0.0	90.0	0.0	10.0	50.0	30.0	20.0
	Potato	08/12/2006		20	80.0	20.0	0.0	85.0	10.0	5.0	65.0	30.0	5.0
	Potato	11/05/2007		12	91.7	8.33	0.0	100	0.0	0.0	75.0	25.0	0.0
	Potato	18/04/2008	CptL8	32	96.9	0.0	3.12	100	0.0	0.0	62.5	21.9	15.6
	Potato	28/09/2008	CptE8	32	62.5	37.1	0.0	84.4	0.0	15.6	78.1	18.8	3.12
Total				397	35.0	51.9	13.1	80.4	2.77	16.9	71.5	18.9	9.57
Manouba	Peach	16/06/2005		12	16.7	50.0	33.3	83.3	0.0	16.7	66.7	25	8.33
	Peach	28/03/2006		24	0.0	16.7	83.3	95.8	4.16	0.0	20.8	79.2	0.0
	Potato	17/04/2006		12	0.0	83.3	16.7	75.0	0.0	15.0	41.7	41.7	16.7
Total				48	4.16	41.7	54.2	87.5	2.08	10.4	37.5	56.3	6.25
Jendouba	Peach	06/03/2005	Jpe5	20	25.0	75.0	0.0	85.0	0.0	15.0	55.0	45.0	0.0
	Peach	17/04/2006		24	91.7	8.33	0.0	100	0.0	0.0	100	0.0	0.0
	Peach	22/03/2007	Jpe7	68	86.8	13.2	0.0	95.6	2.94	1.47	91.2	7.35	1.7
	Potato	22/05/2007		12	83.3	16.7	0.0	100	0.0	0.0	100	0.0	0.0
Total				124	77.4	22.6	0.0	95.2	1.61	3.22	87.9	11.3	0.8
Kairouan	Peach	30/04/2005		12	8.33	83.3	8.33	75.0	8.33	16.7	58.3	33.3	8.33
	Peach	10/04/2006	Kpe6	61	6.55	80.3	13.1	55.8	0.0	44.3	93.4	6.55	0.0
	Peach	06/04/2008	Kpe8	65	12.3	86.2	1.53	49.2	1.53	49.2	84.6	12.3	3.07
	Peach	03/12/2008		24	20.8	75.0	4.16	83.3	0.0	16.7	87.5	8.33	4.16
	Peach	02/06/2009		26	38.5	53.8	7.69	92.3	7.69	0.0	100	0.0	0.0
	Potato	06/05/2008	Kpt8	15	13.3	73.3	13.3	46.7	0.0	53.3	86.7	13.3	0.0
	Potato	03/12/2008		12	50.0	50.0	0.0	75.0	0.0	15.0	91.7	8.33	0.0
Total				215	16.7	76.3	6.97	62.8	1.86	35.3	88.4	9.76	1.86

Total	784	34.8	53.3	11.9	78.3	2.29	19.4	76.3	18.0	5.74

n is the number of individuals tested. An asterisk identifies samples analysed for microsatellite variation

535 **Table 2**. Proportion of knockdown resistance genotypes in Tunisian samples of *Myzus*

536 *persicae*

Kdr resistance genotype	Location				Total	Percentage
	Capbon n=516	Manouba n=48	Jendouba n=124	Kairouan n=215		
SSSS	0.35	0.04	0.77	0.16	319	35.3
SRSS	0.33	0.31	0.17	0.4	298	33.0
RRSS	0.10	0.52	0	0.05	91	9.96
RRSR	0.01	0.02	0	0.01	11	1.21
SSSR	0	0	0	0	0	0
SSRR	0	0	0	0	0	0
SRSR	0.01	0.004	0.01	0.004	11	1.10
SRRR	0.17	0.1	0.03	0.35	173	19.2
RRRR	0	0	0	0	0	0

537 *n* is the number of aphids sampled from each site

538

Table 2. Proportion of knockdown resistance genotypes in Tunisian samples of *Myzus*

persicae

Kdr resistance genotype	Location				Total	Percentage
	Capbon n=516	Manouba n=48	Jendouba n=124	Kairouan n=215		
SSSS	0.35	0.04	0.77	0.16	319	35.3
SRSS	0.33	0.31	0.17	0.4	298	33.0
RRSS	0.10	0.52	0	0.05	91	9.96
RRSR	0.01	0.02	0	0.01	11	1.21
SSSR	0	0	0	0	0	0
SSRR	0	0	0	0	0	0
SRSR	0.01	0.004	0.01	0.004	11	1.10
SRRR	0.17	0.1	0.03	0.35	173	19.2
RRRR	0	0	0	0	0	0

541 *n* is the number of aphids sampled from each site

542

543

544 **Table 4.** Percentage of *M. persicae* individuals for suction trap samples with different

545 resistance mechanisms.

546

Date	n	*Kdr* genotype			super-*kdr* genotype			MACE genotype		
		SS	SR	RR	SS	SR	RR	SS	SR	RR
2006	23	65.2	34.8	0.0	74.0	4.34	21.7	78.3	21.7	0.0
2006	24	29.2	62.5	8.33	70.8	4.16	25.0	58.3	41.7	0.0
2007	12	75.0	25.0	0.0	91.7	0.0	8.33	100	0.0	0.0
2007	12	50.0	41.7	8.33	91.7	0.0	8.33	75.0	25.0	0.0
2008	24	20.8	75.0	4.16	75.0	8.33	16.7	87.5	12.5	0.0
2008	24	16.7	70.8	12.5	83.3	4.16	12.5	75.0	20.8	4.16
Total	119	38.7	55.5	5.88	79.0	4.20	16.8	77.3	21.8	0.84

547 *n* is the number of individuals tested

548

549 **Table 5.** Number of genotypes, clonal diversity (G), mean number of alleles per locus and
550 allelic richness per locus for each population. Values of allelic richness were calculated based
551 on a minimal sample size of 3 individuals

	Populations												
	Cpe5	Cpe6	CpeL8	CpeE8	Cpe9	Cpt6	CptL8	CptE8	Kpe6	Kpe8	Kpt8	Jpe5	Jpe7
	N=(10)	N=(15)	N=(7)	N=(3)	N=(18)	N=(6)	N=(3)	N=(8)	N=(21)	N=(24)	N=(4)	N=(18)	N=(16)
No. of multilocus genotypes	9	13	7	2	18	5	3	8	14	23	4	15	10
G	0.9	0.86	1	0.66	1	0.83	1	1	0.66	0.95	1	0.83	0.62
Mean no. alleles per locus	4.6	4.2	3.4	2.8	5.2	4.4	4.0	6.2	6.2	6.4	4.4	5.4	4.4
Allelic Richness per locus													
M49	4.49	4.19	3.50	3.00	3.60	4.15	5.00	3.85	4.11	4.40	4.39	3.19	3.23
M63	1.98	2.13	1.99	2.00	1.93	3.78	4.00	3.34	2.97	2.84	3.25	3.39	3.23
M86	2.52	3.45	2.81	3.00	3.53	3.23	4.00	4.67	3.55	2.70	4.21	3.87	3.32
M40	3.29	1.90	2.77	2.00	3.20	1.99	4.00	3.62	2.16	2.83	3.50	2.36	2.96
Myz9	2.69	1.86	2.83	4.00	2.62	3.71	3.00	3.74	2.74	2.60	3.46	2.13	1.82
Mean	2.99	2.70	2.78	2.8	2.97	3.27	3.8	3.84	2.96	3.07	3.76	2.98	2.91

552

553

554   **Table 6.** Genetic differentiation in microsatellite loci expressed as F_{ST} values for pairs of

555   samples pooled by geographical origin
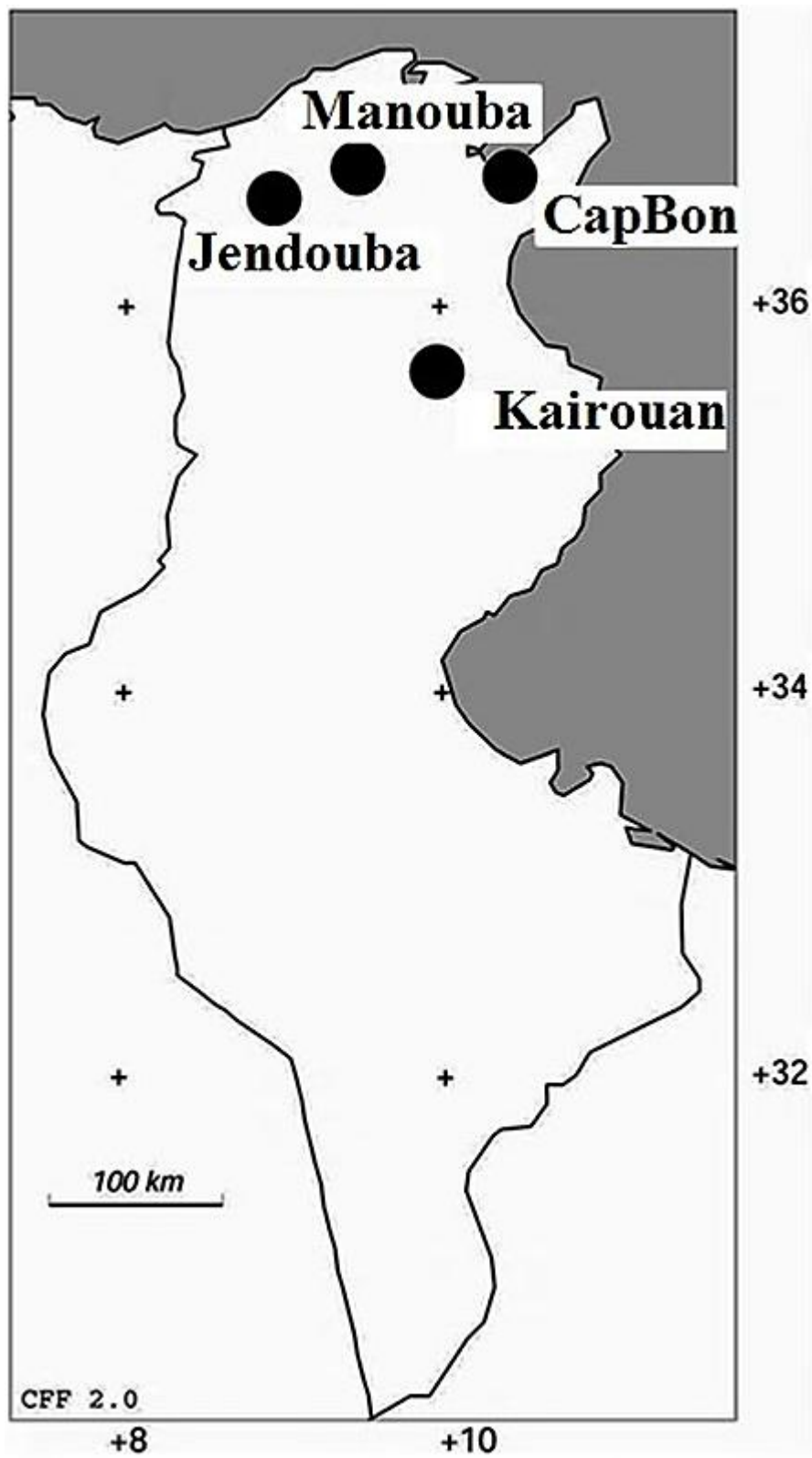
	Cap bon	Jendouba	kairouan
Cap bon	-		
Jendouba	0.040*	-	
kairouan	0.023*	0.071*	-

556   Above F_{ST} value * $P < 0.001$

557

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