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1 **Molecular characterization of genetic diversity within the Africa/Middle East/Asia**
2 **Minor and Sub-Saharan African groups of the *Bemisia tabaci* species complex**

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24 **Running title:** PCR-RFLP for detecting *B. tabaci* diversity

25

26 **Abstract**

27 *Bemisia tabaci* Gennadius is a major plant pest in many agricultural systems worldwide. It is
28 a complex of cryptic species that differ in many ecological respects, including damage-
29 causing potential. Monitoring the genetic composition of *B. tabaci* populations in the field is,
30 therefore, essential for achieving effective control. Sequencing is costly and time-consuming,
31 thus, efficient diagnostic tools must be developed to perform epidemiological studies
32 involving hundreds of individuals. Here, we describe a PCR-RFLP method for identifying all
33 the putative species comprising the Africa/Middle East/Asia Minor group, including those
34 that are the most devastating crop pests. Moreover, intra-specific diversity associated with
35 specific symbiotic bacteria known to manipulate insect host phenotypes can also be detected.
36 Additionally, this method discriminates between the Africa/Middle East/Asia Minor and the
37 Sub-Saharan African groups, which are sympatric in some areas. This simple, reliable and
38 cost-effective diagnostic tool is ideal for the rapid analysis of a high number of individuals
39 and, thus, has potential applications in field contexts, where it could provide valuable baseline
40 information for pest management programs.

41
42 **Keywords:** PCR-RFLP, *Bemisia tabaci*, *mtCOI* gene, molecular diagnostic, Sub-Saharan
43 Africa group, MEAM1 species, Mediterranean species, pest management program.
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1. Introduction

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The whitefly *Bemisia tabaci* Gennadius is one of the most important pests worldwide, causing significant damage to food and ornamental crops directly by feeding on sap and, to an even greater extent, indirectly by transmitting many plant-pathogenic viruses (Jones 2003). *B. tabaci* displays considerable genetic and biological variability despite its morphological homogeneity, which has led to the conclusion that it is in fact a complex of morphologically indistinguishable ‘cryptic’ species (Perring 2001; De Barro et al. 2011). The genetic complexity of *B. tabaci* has mainly been investigated using mitochondrial cytochrome oxidase gene (*mtCOI*) sequence data (Frohlich et al. 1999; Boykin et al. 2007). This has led to the identification of several genetic groups known as biotypes, but the use of the term ‘biotype’ is now subjected to debate. The taxonomy and systematics of *B. tabaci* have not yet been completely elucidated, but recently it was proposed that a value of 3.5% *mtCOI* sequence divergence should be used to distinguish 28 putative cryptic species within the *B. tabaci* complex (Dinsdale et al. 2010; Hu et al. 2011; see De Barro et al. 2011 and Liu et al. 2012 for review). Those species fall into 11 major groups (> 11% divergence), which include those previously described as biotypes. In this study, we have adopted the nomenclature proposed by Dinsdale et al. (2010), but we have also included the biotype designation so as to make it easier to relate our study to the previously published literature.

Among the 28 putative species, two stand out as being the most predominant and devastating worldwide, the Middle East - Asia Minor species 1 (commonly known as biotype B; referred to hereafter as MEAM1) and the Mediterranean MED species (which includes the Q, J, L and ASL biotypes). These two species belong to the same Africa/Middle East/Asia Minor major group of *B. tabaci* identified by Dinsdale et al. (2010), which also includes MEAM2 and Indian Ocean IO species (formerly known as the MS biotype). The MED and

70 MEAM1 species have colonized large areas of most continents as a result of
71 introduction/invasion processes (Bedford et al. 1994; Liu et al. 2007; De Barro and Ahmed
72 2011). Their invasive ability is mainly attributable to their having a broad spectrum of host
73 plants, better reproductive and competitive potential, and greater insecticide resistance. In the
74 Mediterranean basin, the MED species predominates. In some areas, it coexists locally with
75 MEAM1. The MED species is common in North Africa, and has spread, probably recently,
76 into West Central Africa and South Africa, where it coexists with the Sub-Saharan Africa
77 indigenous species (also known as the AnSL biotypes; referred to hereafter as SSAF species)
78 (Gueguen et al. 2010; Esterhuizen et al. 2013).

79 The MED species appears to be genetically diverse not only because it includes the Q, J, L,
80 and Sub-Saharan Africa Silverleaf ASL biotypes (De Barro et al. 2011), but also because high
81 genetic variability has been observed within the Q group, leading to the recognition of several
82 cytotypes on the basis of *mtCOI* gene sequencing and symbiotic bacterial infection (Gueguen
83 et al. 2010; Chu et al. 2012). Chu et al. (2012) recognized five subclades, designated as Q1–
84 Q5, four of which correspond to the previously known Q1, Q2, Q3 and ASL genetic groups
85 (Gueguen et al. 2010). These genetic groups display some particular features. First, they have
86 different geographical and host plant ranges. For example, Q1 and Q2 have been sampled at a
87 large geographical scale on a wide spectrum of host plants, whereas Q3 has only been
88 observed in Western Africa on *Lantana camara* and tobacco (Gnankiné et al. 2012). The Q
89 subclades also differ with regard to the composition of the bacterial symbiotic communities
90 they harbor (Gueguen et al. 2010; Gnankiné et al. 2012). Some of these bacteria are known to
91 induce drastic phenotypic change in *B. tabaci*, conferring features such as a fitness benefit
92 (Kontseladov et al. 2008; Himler et al. 2011). Finally, they also differ as regards the

93 frequencies of alleles resistant to pyrethroids and organo-phosphates (Mouton et al.
94 unpublished data).

95 The spread of the MED and MEAM1 species worldwide has had a considerable impact on
96 agriculture (for review, see Oetting and Buntin 1996), and many studies have focused on
97 identifying the mechanisms underlying their invasiveness and the best methods of control.
98 This requires monitoring *B. tabaci* populations to track the evolution of the species
99 composition in the invaded areas. Gene sequencing is costly and time-consuming, therefore,
100 other effective diagnostic tools need to be developed in order to perform epidemiological
101 studies involving hundreds of individuals. One of the various PCR-based methods, PCR-
102 RFLP, consists of digestion with restriction enzymes after a polymerase chain reaction, and it
103 provides a simple, fast and effective way to detect some of the variations in DNA sequences
104 without sequencing. This technique has already been developed for use in *B. tabaci*, mainly to
105 distinguish MEAM1, MED and non-MEAM1, non-MED species (Bosco et al. 2006;
106 Tsagkarakou et al. 2007; Vassiliou et al. 2008; Ma et al. 2009; Shatters et al. 2009).
107 Moreover, other PCR-RFLP protocols have been proposed to detect genetic variability in the
108 Mediterranean basin, but these do not identify all the genetic diversity known to exist in the
109 Africa/Middle East/Asia Minor group (Sartor et al. 2008; Chu et al. 2012). In this study, we
110 describe a PCR-RFLP method that can be used to identify all the putative species that belong
111 to the major groups (MED, MEAM1, MEAM2, Indian Ocean IO species), and also to
112 recognize within-species variations. We also considered the Sub-Saharan Africa group, which
113 is composed of several Sub-Saharan African species (SSAF1–5 species), which are sympatric
114 with the MED species in some localities (Berry et al. 2004; Gueguen et al. 2010; Gnankiné et
115 al. 2012; Esterhuizen et al. 2013).

116

2. Materials and methods

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2.1. Phylogenetic analyses

120 We performed a phylogenetic analysis to determine the genetic diversity within *B. tabaci*
121 populations in the two major groups under consideration in this study, *i.e.*, the Africa/Middle
122 East/Asia Minor and the Sub-Saharan Africa clades. All the *mtCOI* sequences of *B. tabaci*
123 available in Genbank for these two groups were analyzed, and only the sequences of around
124 657 bp with no undefined nucleotides, and no gaps or indels, were used. This gave us 143
125 sequences originating from samples collected worldwide (Table 1). Multiple sequence
126 alignment was carried out using MUSCLE software (Edgar 2004) implemented in CLC DNA
127 Workbench (CLC bio). Only non-redundant sequences were retained to construct the tree; this
128 gave us 25 haplotypes (for each haplotype, the sequence used for the analysis is shown in
129 bold type in Table 1). Phylogenetic analyses were performed using maximum likelihood and
130 Bayesian inferences. The appropriate model of evolution selected with jModeltest v0.1.1
131 (Posada 2008) was *GTR+I+G* for both methods. ML analyses were performed with Phylml v
132 3.0 (100 bootstrap replicates) (Guindon et al. 2010). Bayesian analyses were done using
133 MrBayes v 3.1.2 with 500,000 generations (Ronquist and Huelsenbeck 2003). Results were
134 plotted using Figtree v1.3.1.

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2.2. *In-silico* selection of restriction enzymes

137 Restriction enzymes were selected by *in silico* analyses of the 25 haplotypes using the CLC
138 DNA Workbench 6.0 (CLC Bio) program. CLC sequence viewer was used to predict
139 restrictions sites on a 657-bp fragment of the *mtCOI* gene sequence with 50 restriction
140 enzymes.

141

142 **2.3. PCR-RFLP on field samples**

143 The PCR-RFLP tool developed in this study was tested on almost 1,100 *B. tabaci* individuals
144 originating from France, Spain, Israel, Greece, Togo, Benin and Burkina Faso, and collected
145 from various host plants (Table 2). DNA was extracted using the procedure of Gueguen et al.
146 (2010). Briefly, individual insects were ground in 25 μ L of extraction buffer containing 50
147 mM KCl, 10 mM Tris-base pH 8.0, 0.45% Nonidet P-40, 0.45% Tween 20 and 500 mg/mL
148 proteinase K. Samples were incubated at 65 °C for 3 h and then at 100 °C for 15 min. A 35-
149 μ L aliquot of pure water was added to this extract, which was then stored at -20 °C until use.
150 An 819-bp fragment of *mtCOI* was amplified with the primers C1-J-2195 and L2-N-3014
151 (Simon et al. 1994). PCR amplifications were performed in a final volume of 25 μ L
152 containing 200 μ M dNTPs, 200 nM of each primer, 1.5 mM of MgCl₂, 0.5 U of *Taq*
153 polymerase (EuroblueTaq, Eurobio, France) and 2 μ L of DNA template. The cycling profile
154 consisted of an initial denaturing phase at 95 °C for 2 min, followed by 35 cycles consisting
155 of: 94 °C for 1 min (denaturing), 50 °C for 1 min (annealing) and 72 °C for 1 min
156 (extension) followed by a final extension phase at 72 °C for 10 min. PCR products (10 μ L)
157 were digested with XapI, and another 10 μ L was digested with BfmI (10 UI) at 37 °C for 3 h.
158 The products obtained were then separated by electrophoresis on a 2% agarose gel at 100 V
159 for 1 h and visualized by ethidium bromide staining. In parallel, PCR products were directly
160 sequenced from 120 individuals.

161

3. Results

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164 **3.1. Phylogenetic structure**

165 The phylogenetic tree obtained with the 25 selected haplotypes is consistent with the most
166 recent *B. tabaci* phylogenies available in the literature (Dinsdale et al. 2010; De Barro et al.
167 2011). Since the ML and the Bayesian analyses led to substantially the same topology, we
168 present only the ML analysis (Fig. 1). Genetic variability was observed within the major
169 groups and species recognized by De Barro et al. (2011). Fifteen haplotypes were identified in
170 the Africa/Middle East/Asia Minor group, and seven in the Sub-Saharan Africa group.
171 Genetic diversity was also found within species, with up to nine haplotypes in the MED
172 species and four in MEAM1. Polymorphism was lower in the other species analyzed (five
173 haplotypes for SSAF5, three for SSAF1, two for SSAF2 and one for MEAM2, IO, SSAF3
174 and SSAF4), but this may have been due to the limited number of sequences available in
175 Genbank for these species.

176

177 **3.2. In silico PCR-RFLP**

178 Two restriction enzymes were selected after *in-silico* analysis on the basis of their capacity to
179 discriminate between the Africa/Middle East/Asia Minor and Sub-Saharan Africa major
180 groups, and their ability to discriminate within these groups between the putative species
181 described by Dinsdale et al. (2010) and De Barro et al. (2011). Ten and nine profile types
182 were found for *XapI* and *BfmI*, respectively (Fig. 2). Both enzymes were able reliably to
183 distinguish between the two major groups. For *XapI*, we found profile types #1, 2, 5, 8, 9 and
184 10 for the Africa/Middle East/Asia Minor group, and profile types #3, 4, 6 and 7 for the Sub-
185 Saharan Africa major group. For *BfmI*, profiles #1, 2, 4, 5, 6, 8, 9 correspond to the

186 Africa/Middle East/Asia Minor group, while profiles #3 and 7 characterized the Sub-Saharan
187 Africa major group (Fig. 1, Table 1). Seventeen profiles were obtained when simultaneous
188 DNA digestion by both enzymes was performed: 12 for the Africa/Middle East/Asia Minor
189 group and 7 for the Sub-Saharan Africa group (Table 1). Within the Sub-Saharan Africa
190 group, we were unable to distinguish all the SSAF species. In contrast, within the
191 Africa/Middle East/Asia Minor group, PCR-RFLP profiles distinguished all the putative
192 species: profiles 1, 2 and 3 were particular to the MEAM1 species; profiles 5 to 11 were
193 specific to the MED species, and profiles 4 and 12 characterized the MEAM2 and IO species,
194 respectively. In summary, *in-silico* analysis suggests that *XapI* and *BfmI* acting
195 simultaneously offer good potential identification of all four putative species in the
196 Africa/Middle East/Asia Minor group, including the ability to detect within-species diversity,
197 since 12 profiles were obtained for 15 known haplotypes.

198

199 **3.3. PCR-RFLP on field samples**

200 The *in-silico* predictions were checked on field samples belonging to the Africa/Middle
201 East/Asia Minor (MEAM1 and MED species) and the Sub-Saharan Africa (SSAF1 and
202 SSAF2 species) groups. We performed both PCR-RFLP and *mtCOI* gene sequencing on 120
203 *B. tabaci* individuals collected worldwide from several plants (Table 2). For another 959
204 individuals, the identification was done using the PCR-RFLP method only, in order to check
205 the reliability of the method. Digestion with *XapI* and *BfmI* generated six and four distinct
206 restriction patterns in our samples, respectively, differing by the number and/or size of the
207 fragments, making it possible to identify the MEAM1, MED and SSAF species (Fig. 3) as
208 predicted by the *in-silico* analysis (Table 1). Moreover, restriction profiles differentiated
209 haplotypes within the MED species, making it possible to recognize the commonly known

210 ASL, Q1, Q2, Q3 genetic groups previously described as different biotypes or cytotypes,
211 which harbor different bacterial endosymbionts. Fragments of less than 50 bp were not
212 detected, because they were too small to be visualized by electrophoresis in a routine agarose
213 gel assay. Moreover, the size of the bands observed for some restriction profiles deviated
214 slightly from predictions; this was because the *in-silico* analysis was performed on 657 bp,
215 which is only a part of the 867-bp amplicon obtained with the primers used to amplify the
216 *mtCOI* gene in *B. tabaci* (C1-J-2195 and L2-N-3014; Simon et al. 1994).

217 For all 120 individuals in which biotype identification was carried out using both standard
218 *mtCOI* gene sequencing and PCR-RFLP assay, the same results were obtained by both
219 methods (Table 2). Moreover, we did not detect any unexpected restriction profiles for the
220 959 other individuals screened by PCR-RFLP only. This implies there was no other sequence
221 variation at the *XapI* and *BfmI* restriction sites than those detected by the *in silico* analysis.

222

4. Discussion

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225 In this paper a simple diagnostic tool, based on a PCR-RFLP method on the *mtCOI* gene was
226 used:

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228 1) To reliably distinguish between the two major groups of *B. tabaci* that coexist in
229 sympatry in some places in the Mediterranean basin and Africa (Gueguen et al. 2010;
230 Gnankiné et al. 2012; Esterhuizen et al. 2013): the Africa/Middle East/Asia Minor group,
231 which includes the two most invasive members (B and Q biotypes), and the Sub-Saharan
232 Africa group, which is regarded as the basal clade of *B. tabaci*.

233

234 2) To detect all the putative species defined in the Africa/Middle East/Asia Minor group by
235 De Barro et al. (2011) and Dinsdale et al. (2010). These species include MEAM1 (formerly
236 referred to as biotype B) and MED (biotype Q), which are widespread and considered to be
237 the ones that cause the most damage (Oetting and Buntin 1996). In many places, displacement
238 of MEAM1 by MED has been observed (Horowitz et al. 2005; Crowder et al. 2010), and the
239 mechanism(s) underlying this phenomenon have been investigated for many years; this
240 requires monitoring *B. tabaci* populations to track the evolution of the species composition in
241 these areas. One explanation for this displacement could be the fact that these two species
242 differ in their susceptibility towards various insecticides. Indeed, it has been demonstrated
243 that the use of neonicotinoids or pyriproxyfen positively selects for the MED species, which
244 exhibits greater resistance to these insecticides than the MEAM1 species (Horowitz et al.
245 2005).

246 Genetic diversity at the mitochondrial level has also been detected at a finer scale, *i.e.*,
247 within species. This PCR-RFLP protocol makes it possible to identify entities previously
248 recognized as the Q1, Q2, Q3 and ASL genetic groups on the basis of *mtCOI* gene
249 sequencing. While it is still not completely clear whether these groups are of any biological
250 significance, some arguments suggest that this distinction should be taken into account. First,
251 these haplotypes harbour particular symbiont communities that can potentially modify a
252 number of biological traits in their host (Chiel et al. 2007; Gueguen et al. 2010; Gnankine et
253 al. 2012). Second, all the genetic groups of MED species are structured geographically
254 (Gueguen et al. 2010; Gnankiné et al. 2012), so that tracking them provides a way of
255 identifying the invasion routes of *B. tabaci*. Lastly, they are associated with biological
256 features, such as host plant use (Gnankiné et al. 2012), and the frequency of insecticide-
257 resistant alleles (Mouton et al., unpublished data). This further suggests that the mitochondrial
258 diversity could also be associated with nuclear variation and possible genetic isolation. This
259 last point is important because species definition by Dinsdale et al. (2010) is based on *mtCOI*
260 divergence, which means that some so-far unidentified, genetically isolated entities may exist
261 at a finer phylogenetic scale. In a recent study, Chu et al. (2012) found five haplotypes within
262 the MED species by *mtCOI* sequencing. However, the PCR-RFLP method they developed for
263 the *mtCOI* gene using the restriction enzyme *VspI* only distinguished one haplotype, which
264 corresponds to the commonly known Q1 biotype, but the method was unable to detect the
265 other four. The tool we developed here can be used to identify four out of the five *mtCOI*
266 haplotypes described in the MED species. It can thus detect diversity at a very fine scale,
267 which may make it very useful in population studies and may have major implications for
268 pest management programs.

269 Levels of resistance and resistance mutation frequencies to insecticides differ between *B.*
270 *tabaci* species (Horowitz et al. 2005; Wang et al. 2010; Alon et al. 2006, 2008) but also
271 within species as has been observed within the MED species (Mouton pers. com.) Therefore,
272 to be effective, strategies developed to control this pest must differ according to the local *B.*
273 *tabaci* composition and the chemical classes of insecticides used. Monitoring the evolution of
274 genetic diversity is, thus, clearly required for effective control strategies of this pest. The tool
275 developed here has already been successfully used for describing the diversity of *B. tabaci*
276 genetic groups in western Africa (Gnankiné et al. 2012; Gnankiné et al. 2013; Mouton, pers.
277 com.) and could be useful to monitor the dynamics of *B. tabaci* populations in relation to
278 insecticide resistance in countries, like west African countries, where insecticides are widely
279 used and lead to high levels of resistance (Houndété et al. 2010).

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289

290

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393

Figure legends

394 **Figure 1:** *mtCOI* gene-based phylogeny of the Africa/Middle East/Asia Minor and Sub-
395 Saharan Africa major groups of *B. tabaci* using maximum likelihood (ML) analyses.

396 Bootstrap values are shown at the nodes for ML analysis, followed by the corresponding
397 posterior probabilities obtained by Bayesian inferences. Sequences were retrieved from
398 Genbank. *Bemisia afer* was used as the outgroup. The analysis was based on a 657-bp
399 sequence. Biotypes and access numbers are indicated at each branch, as is the type of profile
400 obtained with each enzyme (*XapI*/*BfmI*). Frames indicate the specific profiles that can be used
401 to distinguish between the sequences (for example, profiles #10 and #2 obtained with *XapI*
402 are specific to the MEAM1 species, whereas restriction with both enzymes is required to
403 identify Q1, Q2, Q3, and L2).

404

405 **Figure 2:** *In-silico* restriction profiles obtained with *XapI* (A) and *BfmI* (B). The sizes of the
406 bands corresponding to the ladder are indicated (bp).

407

408 **Figure 3:** PCR-RFLP assays with *XapI* (A) and *BfmI* (B).

409 The sizes of the different bands obtained, and the sizes (bp) of the bands corresponding to the
410 ladder are indicated on the left and the right of the figure, respectively.