A multiplex PCR to differentiate the two sibling species of mosquitoes *Ochlerotatus detritus* and *Oc. coluzzii* and evidence for further genetic heterogeneity within the Detritus complex

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**ABSTRACT**

Internal transcribed spacer regions of ribosomal DNA were sequenced, and species-specific primers were designed to simplify the identification of two morphologically similar species of the Detritus complex, *Ochlerotatus detritus* and *Ochlerotatus coluzzii*. Each newly designed primer was able to amplify a species-specific fragment with a different size. Samples from France and Germany were successfully tested. This new tool prompts for bio-ecological studies to refine basic knowledge on the bionomics of this species complex, towards a better control and prevention of ensuing nuisances. Moreover, ITS2 sequencing revealed the existence of (1) two distinct haplotypes of *Oc. detritus* that are sympatric and widely distributed along the French Atlantic and Mediterranean littorals and (2) a specific haplotype in mosquitoes sampled from Tunisia, raising the question of the taxonomic status of this North-African population.

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1. Introduction

The Detritus complex is composed of two morphologically similar species of mosquito *Ochlerotatus detritus* (former species B, described as *Culex detritus* Haliday, 1833) and *Ochlerotatus coluzzii* (former species A, described as *Aedes* (*Ochlerotatus*) coluzzii Rioux, Guilvard and Pasteur, 1998) [Rioux et al., 1998]. In the tribe Aedini, the taxon *Ochlerotatus*, formerly a subgenus of *Aedes*, was restored to genus rank in 2000 and the species of the Detritus complex are maintained in that genus [Reinert, 2000]. There is no subgenus so far assigned (subgenus uncertain). Immature stages of the Detritus complex use brackish breeding sites. It is largely distributed from United Kingdom to Mongolia, including the whole Mediterranean area, Iran and Saudi Arabia. *Oc. detritus* also occurs in southern Scandinavia (Northern and Baltic Sea coasts) and *Oc. coluzzii* is known from Portugal (see Schaffner et al., 2001 for references related to geographic distribution). The two species of the complex are known to be sympatric in France, Morocco, and Tunisia (Pasteur et al., 1977, 1978; Rioux et al., 1998). The two species exhibit various preferences in their bio-ecology. *Oc. detritus* prefers low-salinity breeding places, is anautogenous (*i.e.* necessitates blood-feeding to maturate a progeny) and eurygamous (needs large space for mating). On the other hand, *Oc. coluzzii* thrives in hypersaline environments such as salt marshes, is possibly autogenous (*i.e.* blood feeding needed to maturate a progeny) and eurygamous (able to mate in confined volume) (Agoulon et al., 1999; Rioux et al., 1998; Roubaud, 1943; Verdier, 1978; Veronesi et al., 2012). However, larvae of the two species have been recorded in the same breeding place. Despite the heavy nuisance these mosquitoes cause, no reliable and straightforward mean exists to differentiate indisputably the two species, except the historical iso-enzymatic techniques that permitted their description in the seventies (Pasteur et al., 1977). Using novel primers based on ITS2 rDNA sequence, we developed a single-step multiplex polymerase chain reaction (PCR) to clearly differentiate the two species. This new method was then evaluated with immature and adult specimens collected in various European
and North African localities. Its specificity was tested against other mosquito species.

2. Materials and methods

2.1. Mosquito collection

Mosquitoes were collected at the larval and adult stages in 10 locations in Western Europe and one location in North Africa (Table 1). Specimens (larvae and some adults) were preserved in ethanol 95%; other adults were conserved dried.

2.2. DNA extraction, amplification and sequencing

Genomic DNA was extracted from single whole larvae or single adult mosquitoes with 2% CTAB and re-suspended in 20 μl of water (Delatte et al., 2011).

Preliminary attempts to amplify the mitochondrial COI gene using the primers and protocols of Rey et al. (2001) were unsuccessful.

The extracted DNA was then successfully used as a template for sequencing of the mitochondrial NADH dehydrogenase subunit 5 (ND5) gene (ND5F: 5'-TCTTTAGAATAAATCCGCGC-3'/ND5R: 5’-GTCTTGCCTTTAGCTTTCTTC-3’) and the two nuclear ribosomal genes D3 (D3a: 5’-GACCCGTCTTAGAACACCGA-3’/D3b: 5’-TCCGAAAGAACAGCTGTTCT-3’) and ITS2 (ITS2A: 5’-TGGTGAATCGAAGCAACAT-3’/ITS2B: 5’-TAGCTTTAATCCGGGTT-3’) using previously described primers sets and sequencing protocols (Kengne et al., 2003; Le Goff et al., 2012; Kamgang et al., 2011).

Briefly, all PCR reactions were performed in 25 μl final volume, including 4 μl of DNA extraction (diluted at 1/50), 1X buffer (QIA-GEN), 1.5 mM of MgCl2, 0.2 mM of each dNTP mix (5 mM), 10 pMole of each primer and 1 Unit of Qiagen Taq DNA Polymerase (5 μU). PCR amplification steps included initial denaturation at 94 °C for 2 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing (52 °C for ND5 and ITS2; 55 °C for D3) and 45 s extension at 72 °C, and a final elongation step at 72 °C for 10 min. After amplification, 10 μl of the PCR products were analyzed by electrophoresis onto a 1.5% agarose gel containing 0.5 μg/ml Ethidium Bromide. The remaining PCR products were purified using AMPure PCR kit (Agencourt, Beverly, MA) and used for sequencing in both directions with the previous forward and reverse primers. Sequences were aligned using CLUSTAL X ( Higgins and Sharp, 1988). Genetic distances between haplotypes were computed using MEGA V5.05 (Tamura et al., 2011), excluding alignment gaps and using the pairwise deletion option with Kimura 2-parameters correction. A maximum-likelihood phylogenetic tree was drawn using MEGA.

2.3. Isoenzymatic identification

Iso-enzymatic polymorphism was investigated at the diagnostic loci, α-glycerophosphate dehydrogenase (αGpd) and glutamate-oxaloacetate transaminase 2 (Got2) on single adult mosquitoes, according to the protocols of Pasteur and collaborators (1977). Prior to mosquito grinding, 2–3 legs were dissected out and used as a source of genomic DNA to validate the multiplex PCR assay (below).

3. Results and discussion

3.1. Analysis of ND5, D3 and ITS2 sequences

(Table 2) ND5. Sequencing of the mtDNA ND5 region in 23 specimens revealed the existence of six haplotypes that differed at 10 SNPs. One of these haplotypes was shared between Oc. detritus and Oc. coluzzii. The length of the sequenced fragments was 424 bp. Sequences have been deposited in GenBank database under the following accession numbers: Oc. coluzzii h1: KJ661032; Oc. detritus h2: KJ661034; Oc. detritus h3: KJ661035; Oc. detritus h4: KJ661036 and Oc. coluzzii h4: KJ661033 that have identical sequences; Oc. detritus h5: KJ661037; Oc. detritus h6: KJ661038.

D3. Sequencing of the D3 domain of the 28S rDNA in 40 specimens revealed the existence of a single haplotype, without variation across a total length of 370 bp. Sequences have been deposited in GenBank database under the following accession numbers: Oc. coluzzii: KJ622045; Oc. detritus: KJ622046 that have identical sequences.

ITS2. Sequencing of the rDNA ITS2 region in 86 specimens revealed the existence of four haplotypes that differed at 16 SNPs (Fig. 1). The length of the sequenced fragments varied between 360 and 366 bp. Sequences have been deposited in GenBank database under the following accession numbers: Oc. detritus h1:
KJ661028; Oc. detritus h2: KJ661029; Oc. coluzzii h3: KJ661030; Oc. detritus h4: KJ661031. Importantly, the 76 specimens from Europe referred to three haplotypes (h1–3, Fig. 1) whereas the 10 specimens from Tunisia presented a unique haplotype (h4, Fig. 1).

Fig. 2 shows an unrooted maximum-likelihood phylogenetic tree based on ITS2 sequence polymorphism. The Tunisian haplotype (h4) occupies an isolated location in the tree, whereas the three European haplotypes (h1–3) cluster together. A rooted tree using Stegomyia albopicta and/or Stegomyia aegypti sequences did not change the tree topology (data not shown). The large genetic divergence of the Tunisian haplotype may reflect increased genetic drift in geographically isolated populations in a genomic region known to experience rapid concerted evolution (Collins and Paskewitz, 1996). However, this finding also questions the taxonomic status of the Tunisian population that will require further investigation.

3.2. Species assignment through the initial electrophoresis method

Nine specimens (7 from Gard, France and 2 from Pyrénées-Orientales, France) were identified to species through enzyme...
characters determined by starch-gel electrophoresis analysis, the only available method to distinguish the two species of the Detritus complex. Two specimens from Gard presented the variant Got2 ‘slow’ and the 7 remaining specimens presented the Got2 ‘fast’ allele. The 2 specimens with Got2 slow allele presented the variant \( \alpha \text{-Gpd}^{100} \) and were therefore identified as Oc. detritus according to (Pasteur et al., 1977). Six specimens with Got2 ‘fast’ presented the genotype \( \alpha \text{-Gpd}^{140}, \) and 1 specimen with Got2 ‘fast’ presented the heterozygous genotype \( \alpha \text{-Gpd}^{140-180}, \) both genotypes corresponding to Oc. coluzzii. Sequencing of the rDNA ITS2 region in these specimens revealed that the two Oc. detritus specimens presented haplotype h1, whereas the seven Oc. coluzzii specimens presented haplotype h3. This distribution 0 vs. 2 and 7 vs. 0 has a significant Fisher’s exact probability of 0.028, highlighting the correspondence between enzyme profiles and ITS2 haplotypes.

3.3. Allele-specific PCR

Nucleotide sequence differences in the ITS2 region of the European specimens were used to design PCR primers for the development of an allele-specific PCR assay for rapid identification of sympatric populations of Oc. detritus and Oc. coluzzii. Primer selection was based on the principles described in (Cohuet et al., 2003; Kengne et al., 2003, 2009; Ndo et al., 2013). A universal forward primer (CoDeUN) that anneals to the conserved 5’-end of the ITS2 in the two species was designed, with two reverse primers OcDe and OcCo that specifically anneal to Oc. detritus and Oc. coluzzii DNA, respectively (Table 3). Within the pool of 86 specimens sequenced for ITS2 region, there is no natural variation in the annealing region of the three primers. The size of the diagnostic band is 219 bp for Oc. coluzzii and varies between 259–263 bp depending on the haplotypes for Oc. detritus (Fig. 1).

Various PCR conditions were tested in order to optimize amplification. The ideal mixture composition for diagnostic PCR was 1.5 mM MgCl\(_2\), 0.2 mM each dNTP (Eurogentec), 10 pmol of each primers, 2.5 μl 10X Taq Buffer, 1U Taq Polymerase (Diamond, Eurogentec), and 5–10 ng of template DNA in 25 μl of final reaction volume. The PCR conditions included an initial denaturation step at 95 °C for 3 min, followed by 32 cycles of 30 s at 95 °C, 45 s at 50 °C, 45 s at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 2% agarose gel (Fig. 2).

To test the general application of the method, 171 field-collected specimens from France and Germany were analysed. The results (Table 1) were largely attempted on the basis of previous studies, which reinforce the confidence in the validity of the PCR assay: (1) both species are sympatric on the French Mediterranean littoral, especially in the departments Loire-Atlantique and Charente-Maritime, and (3) the sole Oc. detritus is observed in north-eastern France (Alsace) and northern Germany.

Using this PCR protocol, the 10 specimens from Tunisia showed a band at 263 bp and can be classified as Oc. detritus. Because both Oc. detritus and Oc. coluzzii have been reported from North Africa (Pasteur et al., 1978; Rioux et al., 1998; Brunhes et al., 2000; Trari et al., 2002), further investigation of the genetic polymorphism and bio-ecological attributes of these Tunisian populations are warranted.

3.4. Validation of the assay

Under the allele-specific PCR conditions, no amplification product was obtained when DNA from Ochlerotatus caspius, Ochlerotatus berlandii, Ochlerotatus dufouri, Ochlerotatus sensu auctorum frayeri, Aedimorphus fowleri, Aedimorphus sp. A (Le Goff et al., 2012), Aedimorphus albopcephalus, Stegomyia aegypti, Stegomyia pia, Stegomyia albopicta, Fredwarsius vittatus and Orthopodomyia pulcripalpis were used as a template. We can therefore conclude that the primers are specific to the Detritus complex.

No heterozygote (Got2 ‘slow-fast’) have been reported among 4,441 specimens tested from Southern France (Pasteur et al., 1977) and among 530 specimens tested from Western France (Brutus et al., 1994) suggesting that the species Oc. detritus and Oc. coluzzii do not interbreed in nature. However, this possibility cannot be fully ruled out. The DNAs from Oc. detritus (ITS2 haplotype h1) and Oc. coluzzii (ITS2 haplotype h3) were artificially mixed to simulate artificial/putative hybrids and tested in the following proportion: 1/1; 1/3; 3/1; 1/9; 9/1. In any case, using the 3 primers together, the sole band of ‘detritus’ was observable on the gel, signifying probably a better affinity of OcDe over OcCo for their target DNA sequence at an annealing temperature of 50 °C in agreement with a lower melting temperature for the latter (50.0 °C vs. 46.0 °C, respectively, Table 3). However, the ‘coluzzii’ band at 219 bp was rescued in all cases when primer OcDe was omitted and MgCl\(_2\) concentration was increased to 2 mM. We recommend such con-

Table 3

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>( T_m )</th>
<th>Species</th>
<th>Size PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoDeUN</td>
<td>AGTACGAGTGACATTTTTTGGAG</td>
<td>46.6 °C</td>
<td>Ochlerotatus coluzzii</td>
<td>219 bp</td>
</tr>
<tr>
<td>OcCo</td>
<td>CACATGAAACCCCTTC</td>
<td>46.0 °C</td>
<td>Oc. detritus haplotype 1</td>
<td>259 bp</td>
</tr>
<tr>
<td>OcDe</td>
<td>AATGGGAATGGCTGGATCC</td>
<td>50.0 °C</td>
<td>Oc. detritus haplotype 2</td>
<td>257 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oc. detritus haplotype 4</td>
<td>263 bp</td>
</tr>
</tbody>
</table>

\( T_m \): melting temperature.

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trol PCR to be used when specific studies are conducted in areas where the two species are known to co-occur.

3.5. Type deposition

Because the holotype and paratypes of *Oc. coluzzii* are no longer available (Antoine Rioux, pers. com.), a neotype, adult male collected in the type-locality (Salins-de-Giraud, Bouches-du-Rhône, France) was designated by PCR using a midleg and deposited in the Collection d’Arthropodes d’Intérêt Médical (ARIM), Laboratoire de taxonomie des vecteurs, Institut de Recherche pour le Développement, Montpellier, France.

In conclusion, we developed a multiplex PCR-based assay to distinguish two heretofore difficult to identify species *Oc. detritus* and *Oc. coluzzii* in areas where they are sympatric. The validity of the assay outside Western Europe is subject to caution and further studies are needed to explore the ecology and genetics of these species in other European and Mediterranean countries where they have been reported. As our results suggest, it is likely that additional genetic heterogeneity exists within the Detritus complex, which are both of taxonomic and biological interest. Nonetheless, the new PCR tool we propose will facilitate the study of life traits to refine basic knowledge on the bionomics of each species in Europe, an obliged pathway towards a better control and prevention of ensuing nuisances.

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