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Characterization of two cryptic species, *Culicoides stigma* and *C. parroti* (Diptera: Ceratopogonidae), based on barcode regions and morphology

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ABSTRACT: Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are insect vectors of economically important veterinary diseases such as African horse sickness, bluetongue, and Schmallenberg virus. The identification of *Culicoides* based on morphological features can be difficult. Three species of biting midges, *Culicoides nubeculosus*, *C. stigma*, and *C. parroti* have emerged in the laboratory from mud collected around watering troughs on a farm in northern France. Emerging *Culicoides* were characterized morphologically and molecularly using molecular markers. The closely related species *C. stigma* and *C. parroti* showed highly divergent sequences for both mitochondrial (cytochrome B and cytochrome oxidase I) and ribosomal DNA first internal transcribed spacer. A RFLP based on a single restriction using the same enzyme (HaeIII) for both cytochrome C oxidase I and cytochrome B is proposed to identify these species. *Journal of Vector Ecology* 38 (2): 260-265. 2013.

Keyword Index: *Culicoides parroti*, *Culicoides stigma*, mitochondrial DNA, ribosomal DNA, sympatry, PCR-RFLP, France.

INTRODUCTION

Biting midges of the genus *Culicoides* Latreille 1809 (Diptera: Ceratopogonidae) are among the world's smallest haematophagous flies, measuring from 1 to 3 mm in size, and are the vectors of orbiviruses such as bluetongue virus (BTV), African horse sickness virus, or Schmallenberg virus (SBV). Outbreaks of BTV and SBV have been recorded since 2006 and 2011, respectively, in France and have caused severe losses in cattle and sheep. However, the identification of *Culicoides* based on morphological features can be difficult. To solve this problem of identification, several techniques have been developed, including morphometric tools (Pagès and Sarto 2005, Pagès et al. 2009, Augot et al. 2010, Muñoz-Muñoz et al. 2011), use of molecular markers such as Cytochrome oxidase subunit I (COI) of the mitochondrial DNA (mtDNA) (Nolan et al. 2007); first and second internal transcribed spacers (ITS1 and ITS2) of the ribosomal DNA (rDNA) (Cêtre-Sossah et al. 2004, Perrin et al. 2006, Gomulski et al. 2006), and an Interactive Identification Key (IIKC) based on morphological items (Mathieu et al. 2012). This database includes 60 descriptions (i.e., wings, spermathecae, eyes, mandible/maxille, cibarial armature, posterior pharynx armature, palpus, antennae, legs). Success rates differed significantly between users and between levels of experience and ranged from 35.1% to 81.1% (Mathieu et al. 2012). The use of COI barcodes as a tool for species identification of biting midges can differentiate 95% of species studied (Ander et al. 2012). Misidentification of disease vectors has important epidemiological implications. Thus, the knowledge of which species could act as disease vectors, as well as their correct identification, remains essential to assess the real risk for

disease transmission into disease-free areas.

In France, two very closely related species, *Culicoides parroti* Kieffer, 1922 and *Culicoides stigma* Meigen, 1818 belonging to the subgenus *Monoculicoides*, show overlapping characters. These two species live in sympatry in the country. Their morphological specific identification is difficult for non-specialists using a microscope and impossible based on wing patterns using a stereomicroscope. The goal of the present study was to morphologically identify *Culicoides parroti*, *C. stigma*, and *C. nubeculosus*, that emerged in the laboratory from mud collected in the French Ardennes, and to provide molecular tools for their identification. From these three species, only *C. nubeculosus* has been proven to biologically transmit BTV to date (Jennings and Mellor 1988). Three different molecular markers were tested for this purpose: i) rDNA ITS1; ii) the mtDNA COI serving as 'gold standard' for DNA barcode used in routine for *Culicoides* studies, and iii) a part of the cytochrome b (cyt b) of the mtDNA. The latter was chosen because of its usefulness at the taxonomic level for many other groups of insects, such as phlebotomine sand flies, and because it is commonly used in our laboratory (Moin-Vaziri et al. 2007, Randrianambinintsoa et al. 2012). The suitability of Cytb and COI sequences to provide the basis of a species-diagnostic polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was also assessed.

MATERIALS AND METHODS

Study site and selection of specimens

In 2008 over a four-month period, mud and dung were collected in and around a farm (49°53'77"N, 4°19'36"E) in the Ardennes. Soil samples rich in organic matter were randomly collected early in the morning around water troughs used by cattle and sheep in the field. These samples were put into plastic boxes that were then labelled. Samples were transferred to the laboratory and monitored in individual netted cages in order to detect emerging *Culicoides*. The laboratory temperature ranged from 18° to 21° C and the flow of water into the plastic boxes was kept constant. The cages were examined daily and adult *Culicoides* were transferred into small vials containing 96% ethanol. The head, wings, and genitalia of individual biting midges were cut off in a drop of ethanol, cleared in boiling Marc-André solution, and mounted between slide and cover slide. The corresponding thorax for each specimen was stored in a vial at -20° C before DNA extraction.

Three species (*C. stigma*, *C. parroti*, and *C. nubeculosus*) emerged from the mud and were morphologically identified and separated using their wing patterns according to the key of Delécolle³. Because of the similarity of the morphological characters of their wings, males and females were identified as *C. stigma* / *C. parroti* by specific genital characters (Figure 1).

Molecular analyses

Genomic DNA was extracted from 14 individuals *Culicoides* specimens (six *C. parroti*, six *C. stigma*, and two *C. nubeculosus*) according to the manufacturer's instructions (Qiagen). We used the primers CulitotalF (5'-GACGCTTATTAATATAGTTC-3') and CulitotalR (5'-TGCGGTCTTCATCGACCCAT-3') designed by Cêtre-Sossah et al. (2004) and Gomulski et al. (2006), respectively. Their combination amplifies a fragment including ITS1, 5.8S, and ITS2. The primers Culitotal F and Culitotal R were used with the following conditions: an initial denaturation step at 94° C for 3 min, followed by 35 cycles of denaturation at 94° C for 1 min, annealing at 56° C for 1min and extension at 68° C for 1 min, and final extension at 68° C for 10 min.

COI was amplified using the primers C1J1718 and C1N2191 as indicated by Dallas et al. (2003). PCR cycling conditions were: an initial denaturation step at 95° C for 15 min, then five cycles at 95° C for 40 s, 45° C for 40 s, 72° C for 1 min, followed by 45 cycles at 95° C for 40 s, 50° C for 40 s, 72° C for 1 min, and a final extension step at 72° C for 20 min. A part of the cytochrome b gene was amplified using the primers N1N-PDR and C3B-PDR as detailed by Bounamous et al. (2008). PCR cycling conditions were: an initial denaturation step at 94° C for 3 min, followed by five cycles of denaturation at 94° C for 30 s, annealing at 45° C for 90 s, and extension at 86° C for 60 s, and then 35 cycles of denaturation at 94° C for

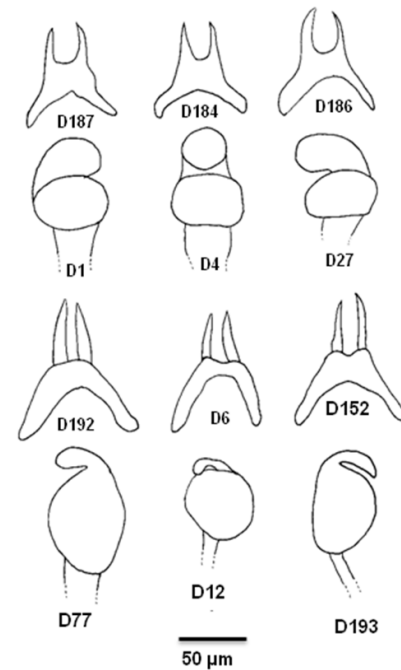


Figure 1. Drawing of parameres and spermathecae of two closely related species of *Culicoides* in Northern France. *Culicoides parroti*: males (D187, D184, D186) and females (D1, D4, D27); *Culicoides stigma*: males (D192, D6, D152) and females (D77, D12, D193).

30 s, annealing at 51° C for 90 s, and extension at 86° C for 60 s and a final extension at 68° C for 10 min.

Direct sequencing of both DNA strands was performed with the primers used for PCR. Sequence alignments were done utilizing ClustalW software (Thompson et al. 1994). The DNA sequence-based analyses were performed using the neighbor-joining (NJ) method (Kimura 2-parameter) with MEGA software version 3.1 (Kumar et al. 2004). For Cytb, only one sequence is available in data base: *C. arakawae* from Japan (GenBank under accession NC009809). For ITS, we have included *C. parroti* from France (AY861153), *C. nubeculosus* from the United Kingdom (AJ417982) and *C. puncticollis* from France (AY861158), and *C. imicola* from Israel (JN408479) to our sampling. For COI, we have added *C. nubeculosus* from Sweden and Denmark (JQ620128 and JQ683275, respectively), *C. puncticollis* from Denmark (JQ683314), *C. riethi* from Denmark (JQ683337, JQ683336), *C. stigma* from Sweden (JQ620229), and *C. imicola* from Spain (AF080540). *C. imicola* have also been processed as an outgroup in ITS1 (Perrin et al. 2006) and COI (Ander et al. 2012), respectively.

Sites for restriction enzymes were predicted for COI and Cytb sequences of *C. parroti* and *C. stigma* using CLC DNA Workbench software version 5.2 (www.clc-genomics-workbench.com-about.com/). A panel of restriction enzymes was tested. *HaeIII* provide an original digestion pattern per species for both mtDNA markers and was selected. PCR-RFLP assays were performed in a 30-µl total volume reaction mix, containing 10 µl of PCR product (from PCR vials), 1 µl

³Delécolle, J.C. Nouvelle contribution a l'étude systématique et iconographique des espèces du genre *Culicoides* (Diptera: Ceratopogonidae) du Nord-Est de la France. PhD thesis. Université Louis Pasteur de Strasbourg, UER Sciences Vie et Terre, 1985.

Table 1. Sampling species in the Ardennes département (France).

Species	No. samples	Sex	Date of mud collection	Date of emergence	Number of days Between collection and emergence
<i>Culicoides parroti</i>	D184	M	12/02/2008	20/02/2008	8
	D186	M	12/02/2008	20/02/2008	8
	D187	M	12/02/2008	20/02/2008	8
	D27	F	12/02/2008	20/03/2008	36
	D1	F	06/03/2008	10/03/2008	4
	D4	F	06/03/2008	10/03/2008	4
<i>Culicoides stigma</i>	D192	M	12/02/2008	10/04/2008	57
	D6	M	06/03/2008	13/03/2008	7
	D152	M	02/04/2008	07/04/2008	5
	D12	F	12/02/2008	13/03/2008	29
	D193	F	12/02/2008	10/04/2008	57
	D77	F	02/04/2008	07/04/2008	5
<i>Culicoides nubeculosus</i>	D16	F	12/02/2008	13/02/2008	29
	D179	M	02/04/2008	07/05/2008	35

of *HaeIII*, and 2 μ l of supplied buffer (Fermentas, Germany). PCR products were digested for 20 min at 37° C. The digested samples were separated by electrophoresis in a 3% agarose gel to produce DNA fragments and were sized by comparison with markers 50 pb ladder and 100 pb ladder (Cliniscience, France).

RESULTS

Mud samples were collected near watering troughs, within 30 m of livestock. A total of 115 adult *Culicoides* specimens made up of 67 females and 48 males emerged. They belonged to three *Monoculicoides* species: *Culicoides stigma* (10♀, 8♂); *C. parroti* (9♀, 9♂), and *C. nubeculosus* (48♀, 31♂). The date of emergence ranged from four to 36 days for *C. parroti*, five to 57 days for *C. stigma*, and 29 to 35 days for *C. nubeculosus* (Table 1). No other species of *Culicoides* emerged from the mud during the sampling period.

The length of PCR products amplified ranged approximately 372 to 537 bp according to the primers. The sequence size, including primer, used to detect sites for restriction enzymes is 574 bp for COI and 544 bp for Cytb. Molecular comparisons after alignment (including Genbank sequences) were based on 400 bp for the ITS-1, 558 bp for the Cyt b, and 472 bp for the COI (including gaps). The sequences have been deposited into GenBank under the accession numbers: for ITS1: *C. nubeculosus* (KF178258- KF178259), *C. parroti* (KF178260- KF178265), *C. stigma* (KF178266- KF178271); for COI: *C. nubeculosus* (KF178272- KF178273); *C. parroti* (KF178274- KF178278); *C. stigma* (KF178279- KF178284); for Cytb: *C. nubeculosus* (KF178285- KF178286), *C. parroti* (KF178287- KF178292), *C. stigma* (KF178293- KF178298). The sequences were compared using the pairwise distance between each group (Table 2) including sequences previously deposited in Genbank.

Analysis of ITS1 sequences

The lengths of ITS1 segments were 372-375 bp for *C. nubeculosus*, 383-423 bp for *C. stigma*, and 396-415 bp for *C. parroti*. The genetic distance pairwise within species showed 99.7%, 99.9%, and 100% homology within *C. nubeculosus*, *C. stigma*, and *C. parroti*, respectively, whereas distance pairwise computed between species showed 87.6% homology between *C. nubeculosus* and *C. parroti*, 91% between *C. nubeculosus* and *C. stigma*, and 91.8% between *C. stigma* and *C. parroti*. The NJ analysis suggested that *C. stigma* is closer to both *C. puncticollis* and *C. nubeculosus* than to *C. stigma* (Figure 2).

Analysis of COI sequences

The lengths of COI segments were 513-529 bp for *C. nubeculosus*, 502-512 bp for *C. parroti*, and 498-524 bp for *C. stigma*. The genetic distance pairwise within species showed 99.6%, 99.8%, and 99.4% homology within *C. nubeculosus*, *C. stigma*, and *C. parroti*, respectively, whereas distance pairwise computed between species showed 83.1% homology between *C. nubeculosus* and *C. parroti*, 80.8% between *C. nubeculosus* and *C. stigma*, and 86.3% between *C. stigma* and *C. parroti*. The NJ analysis suggested that *C. stigma* and *C. parroti* are the two closest species. On another branch, the species *C. riethi* *C. puncticollis* and *C. nubeculosus* are grouped (Figure 2).

Analysis of cytochrome b sequences

The lengths of Cytb segments were 516-519 bp for *C. nubeculosus*, 517-537 bp for *C. parroti*, and 513-525 bp for *C. stigma*. The genetic distance pairwise within species showed 99.8%, 99.7%, and 99.9% homology within *C. nubeculosus*, *C. stigma*, and *C. parroti*, respectively, while distance pairwise computed between species showed 82.8% homology between *C. nubeculosus* and *C. parroti*, 85.3% between *C. nubeculosus* and *C. stigma*, and 84.3% between *C. stigma* and *C. parroti*. The NJ analysis suggested that *C. stigma* is the sister group of *C. nubeculosus* (Figure 2).

Table 2. Estimation of pairwise distance species *C. parroti*, *C. stigma*, *C. nubeculosus*, *C. puncticolis*, *C. imicola*, *C. riethi*, and *C. arakawae* for ITS1 region of the rDNA, the COI, and Cytb domain of the mtDNA, respectively.

	ITS1				COI					Cytb		
	1	2	3	4	1	2	3	4	5	1	2	3
1 <i>C. parroti</i>												
2 <i>C. stigma</i>	0.0082											
3 <i>C. nubeculosus</i>	0.124	0.090								0.173		
4 <i>C. puncticolis</i>	0.108	0.076	0.043							0.147	0.157	
5 <i>C. imicola</i>	0.251	0.272	0.261	0.273						0.212	0.171	0.204
6 <i>C. riethi</i>					0.198	0.137	0.216					

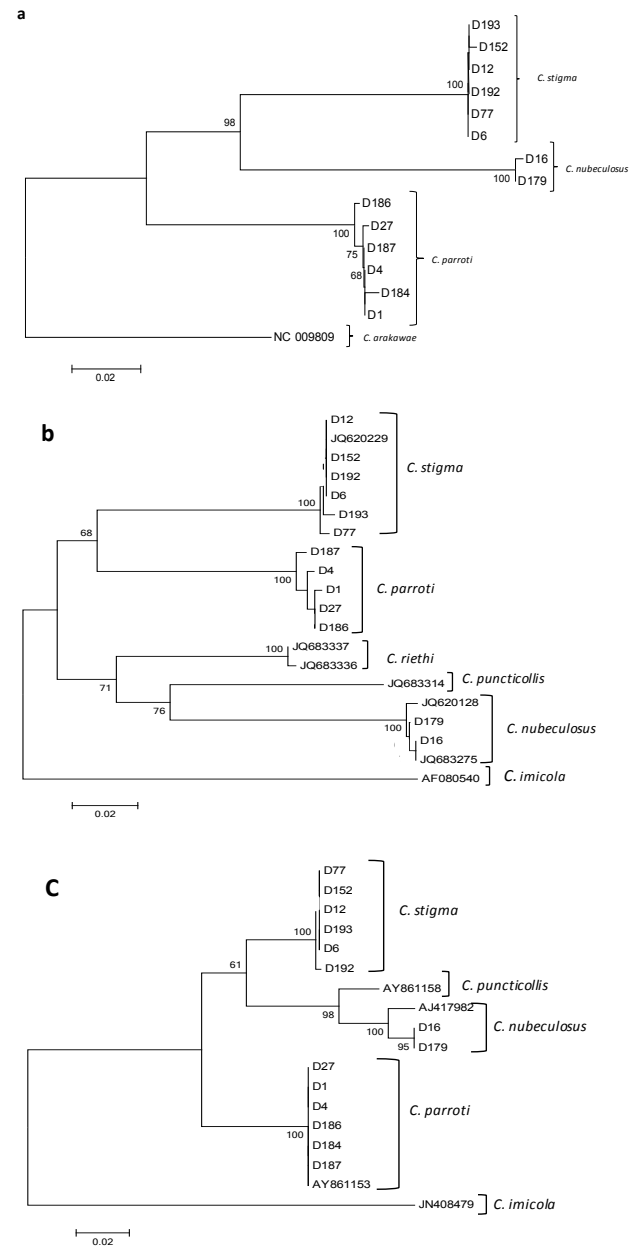


Figure 2. Neighbor-joining trees based on mtDNA (Cytochrome b (a), Cytochrome oxidase I (b) and first internal transcribed spacer rDNA (c) for sequences of *Culicoides nubeculosus*, *C. parroti*, and *C. stigma* from northern France. Bootstrap values (1,000 replicates) are given on the branches.

Although *C. parroti* and *C. stigma* males are closely related morphologically, the mitochondrial and ribosomal molecular markers strongly distinguish the former from the latter (Figure 2). The membership of each sample in the various branches was strongly supported by bootstrap values.

Species-diagnostic restriction enzymes sites

Recognition site for the restriction enzyme (*Hae*III) was identified in the COI and Cytb sequences. It distinguishes *C. parroti* from *C. stigma*. The observed profiles are in agreement with the predicted profiles for COI sequence: *C. parroti*

(469/105 bp) and *C. stigma* (574 bp); and for Cyb b sequence: *C. parroti* (544 bp) and *C. stigma* (226/318 bp).

DISCUSSION

In our study, pupae of *C. nubeculosus*, *C. stigma*, and *C. parroti* coexisted on the same site rich in organic matter, confirming the observations of Uslu and Dik (2006). Among the several genes of interest for the identification of species, the COI and Cytb genes are of interest for the taxonomy of closely related species. COI is the most commonly sequenced marker for *Culicoides* barcoding. To our knowledge, Cyt b has never been used before for specific identification of *Culicoides*. It enabled us to distinguish two closely related species of *Culicoides*. It yielded readily usable sequences (without sequences containing mixtures of two bases at sites) and could serve as a candidate for standardized molecular identification of *Culicoides* in the future. Species assigned to the subgenus *Monoculicoides* by Ander et al. (2012) are confirmed by our study, showing that *C. stigma* is closely related to *C. parroti* as correlated by morphology.

The coding regions (mtDNA) and non-coding internal transcribed spacer (ITS) sequences are useful tools for phylogenetic studies. ITS1 sequences have been shown to be good phylogenetic markers (Perrin et al. 2006). Our study on *Monoculicoides* subgenera confirms the position of *C. nubeculosus*, *C. puncticollis*, and *C. parroti* reported by Perrin et al. (2006). Interestingly, the molecular analysis of ITS1 has grouped *C. stigma* with *C. puncticollis* and *C. nubeculosus* into the same cluster, whereas *C. parroti* remains outside of the clade. COI provides the grouping of specimens within their specific branch, but the relationships between the branches is different. Regarding COI sequences, *C. stigma* and *C. parroti* are the most closely related species. Hybridization between closely related species of insects is known and common for many insects like Phlebotomine sandflies (Pesson et al. 2004). In *Culicoides*, it has been suspected *in natura* for *C. impunctatus* (Ritchie et al. 2004). In our study, the comparison of the mtDNA and rDNA markers sequenced did not suggest any introgression between *C. stigma* and *C. parroti* found in sympatry.

We applied COI-RFLP and Cytb-RFLP as tools for the identification of *Culicoides* species. This technique is rarely used for the identification of *Culicoides* (Linton et al. 2002, Dallas et al. 2003). The characterization of cryptic species could be obtained using PCR-RFLP and its utilization could be more common in the future.

The identification of *Culicoides* based on morphological features is difficult and several cryptic species and species complexes are present. With the IKC (Mathieu et al. 2012), the successful identifications were achieved in an average of 6.6 steps with three steps for *C. parroti* and four for *C. stigma*. No database is available for identification of *Culicoides* males. Our study, combining morphological and molecular identification of *Culicoides* specimens, is thus important for a better understanding of the systematics of biting midges, which are vectors of diseases, and also to initiate molecular ecological studies.

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