Vet. Res. 35 (2004) 325–337

DOI: 10.1051/vetres:2004015

Original article

Molecular detection of *Culicoides* spp. and *Culicoides imicola*, the principal vector of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe

Catherine CÊTRE-SOSSAH[a]*, Thierry BALDET[a], Jean-Claude DELÉCOLLE[b], Bruno MATHIEU[c], Aurélie PERRIN[a], Colette GRILLET[a], Emmanuel ALBINA[a]

[a] CIRAD-EMVT, Campus international de Baillarguet, TA30/G, 34398 Montpellier Cedex 5, France
[b] Université Louis Pasteur de Strasbourg, Musée zoologique, 29 bd de la Victoire, 67000 Strasbourg, France
[c] Entente Interdépartementale pour la Démoustication, 165 avenue Paul Rimbaud, 34184 Montpellier Cedex 4, France

(Received 12 November 2003; accepted 9 February 2004)

Abstract – Bluetongue (BT) and African Horse Sickness (AHS) are infectious arthropod-borne viral diseases affecting ruminants and horses, respectively. *Culicoides imicola* Kieffer, 1913, a biting midge, is the principal vector of these livestock diseases in Africa and Europe. Recently bluetongue disease has re-emerged in the Mediterranean Basin and has had a devastating effect on the sheep industry in Italy and on the islands of Sicily, Sardinia, Corsica and the Balearics, but fortunately, has not penetrated onto mainland France and Spain. To survey for the presence of *C. imicola*, an extensive light-trap network for the collection of *Culicoides*, was implemented in 2002 in southern mainland France. The morphological identification of *Culicoides* can be both tedious and time-consuming because its size ranges from 1.5 to 3 mm. Therefore, an ITS_1 rDNA polymerase chain reaction (PCR)-based diagnostic assay was developed to rapidly and reliably identify *Culicoides* spp. and *C. imicola*. The aim of this work was to set up a rapid test for the detection of *C. imicola* amongst a pool of insects collected in areas at risk for BT. The sequence similarity of the rDNA (nuclear ribosomal DNA), which is greater within species than between species, is the foundation of its utilisation in species-diagnostic assays. The alignment of the 11 ITS1 sequences of *Culicoides* obtained from Genbank and EMBL databases helped us to identify one region in the 5' end and one in the 3' end that appear highly conserved. PCR primers were designed within these regions to amplify genus-specific fragments. In order to set up a *C. imicola*-specific PCR, another forward primer was designed and used in combination with the previously designed reverse primer. These primers proved to be highly specific and sensitive and permitted a rapid diagnostic separation of *C. imicola* from *Culicoides* spp.

molecular detection / *Culicoides imicola* / ITS1 / PCR / bluetongue

1. INTRODUCTION

Bluetongue (BT) and African horse sickness (AHS) are infectious arthropod-borne viral diseases affecting ruminants and horses, respectively. Bluetongue disease is caused by the bluetongue virus (BTV), which is the prototype of the *Orbivirus*

* Corresponding author: catherine.cetre-sossah@cirad.fr

genus within the Reoviridae family [11]. AHSV is another member of this family and genus. Both viruses are transmitted by certain species of biting midges belonging to the *Culicoides* genus (Diptera: Ceratopogonidae) and are maintained in nature through a series of alternating cycles of replication between its *Culicoides* vectors and susceptible hosts [25].

The spread of bluetongue across all continents pantropically (located between 44° N and 35° S) depends mainly upon the distribution and seasonal abundance of the insect vectors. More than 1 200 *Culicoides* species have been identified in the world [3] but only 17 have been connected with BTV transmission [18]. The major vector species are *C. imicola* and *C. bolitinos* in Africa, *C. imicola* in Asia, *C. fulvus* and *C. brevitarsis* in Australia, *C. sonorensis* in North America, *C. insignis* and *C. pusillus* in South and Central America [17, 18].

As with bluetongue, AHSV is limited to geographical areas where the vector *C. imicola* is present, and its spread depends on the occurrence of climatic conditions favouring vector activity. AHSV is endemic in sub-Saharan Africa but periodically makes brief excursions beyond this area, where it has caused major epizootics extending as far as Pakistan and India in the east, and as far as Morocco, Spain and Portugal in the west [4, 20, 21, 23]. More than 300 000 equines died during the great epizootic of 1959–1961 in the Middle-East and South-West Asia [12]. Until recently, the virus had not survived across more than two seasons in any of these epizootic areas. However, the recent outbreak of AHSV in the western Mediterranean basin, which lasted for five years (1987–1991), has forced us to re-assess the situation with greater vigilance.

The explosive outbreaks of BTV in the Mediterranean Basin since 1998 have been fuelled largely by the classical Afro-Asian vector *C. imicola* [1, 19]. It is known that *C. imicola s.l.* is a complex of at least 10 sibling species but for the time being only *C. imicola s.s.* is present in Europe [16]. In the eastern Mediterranean Basin, outbreaks of BTV have occurred in Bulgaria, Serbia, Kosovo, Croatia, Montenegro, northern Greece and Bosnia Herzegovina in regions up to 44° 30' N, which is further north within Europe than has ever been seen before, and where *C. imicola* has not been detected during insect surveys [2, 19]. This strongly suggests that here the virus is being transmitted by other vector species, possibly by *C. pulicaris* and one or more species of the *C. obsoletus* complex. These Palaearctic species or species complexes are widespread and abundant in the region and across most of northern Europe.

As a consequence of the discovery of *C. imicola* on the island of Corsica in October 2000 [7], the subsequent BTV serotype 2 outbreaks in the autumn of 2000 and 2001 [29–31], and the relatively high risk of occurrence of this vector in neighbouring coastal mainland in southern France [28], it was decided to study the *Culicoides* fauna of this region. For this purpose, a large light-trap network was implemented in 2002 in southern mainland France to survey for the possible presence of *C. imicola*. The morphological identification of *Culicoides* can be both tedious and time consuming because its size ranges from 1.5 to 3 mm. Thus the aim of this study was to set up a rapid diagnostic test for the detection of *C. imicola* amongst a pool of insects collected in areas where livestock are thought to be at risk for BT. The study of molecular taxonomy and phylogenetic evolution of this genus should also give interesting results.

Nuclear ribosomal DNA (rDNA) has several unique features which make it an ideal DNA target for systematic studies [6]. It consists of tandemly repeated copies of the transcriptional unit with three gene regions (18S, 5.8S and 28S) separated by transcribed spacers [8]. As shown in Figure 1, the external transcribed spacer (ETS) is located upstream of the 18S gene, with the first (ITS_1), and second (ITS_2), internal transcribed spacers (ITS) located between either the genes 18S and 5.8S, or between

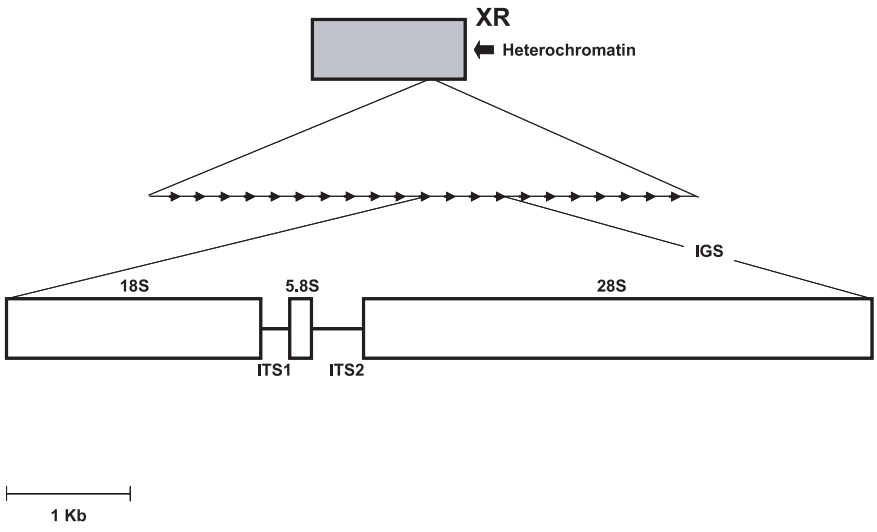


Figure 1. Location and organisation of the ribosomal DNA (rDNA). The rDNA locus consists of one very long array of repeat units (500–700 repeats per genome). Each repeat is 9 kb long and consists of the genes for the 18S, 5.8S, and 28S rDNA (open rectangles). These genes are separated by spacers (solid lines), the internal transcribed spacers (ITS1 and ITS2), and the intergenic spacer (IGS). Adapted from [10].

5.8S and 28S, respectively. It contains regions of varying evolutionary rates, from highly conserved regions (e.g. 5.8S and 18S rRNA genes) to more variable ones (e.g. spacers). Sequence similarity of the rDNA, which is greater within than between species, is the foundation of its utilisation for species-diagnostic assays. A recent study using internal transcribed spacers (ITS) of nuclear ribosomal DNA from *Culicoides* has been performed for a phylogenetic analysis of the genus [15]. Previously, rDNA genetic markers have been used for the identification of mosquitoes (*Anopheles gambiae, An. nili* and *An. minimus* using ITS) [9, 10, 13, 22, 26]. The alignment of the 11 ITS1 sequences of *Culicoides* obtained from Genbank and EMBL databases helped us to focus on one region in the 5' end and one in the 3' end appeared highly conserved. Within these regions, PCR primers were designed to amplify genus-specific fragments. In order to set up a *C. imicola*-specific PCR, another forward primer was designed and used in combination with the previously designed reverse primer. These primers proved to be specific and permitted a rapid diagnostic separation of *Culicoides* spp. from *C. imicola*.

2. MATERIALS AND METHODS

2.1. Traps and collections

We selected 19 sites at risk for the presence of *C. imicola* in (and possible introduction onto) mainland France. These sites are spread at 50 km intervals along the length of the French Mediterranean coast. One collection/night/site, using standard UV-light traps with a suction fan, was performed monthly from April to November 2002. The traps were set, on each night, between 1 h before sunset to around 8.00 am the following morning. They were located outdoors but within 25 m of livestock premises and were suspended from the walls of buildings 1.5–2 m above the ground. A glass collecting beaker containing 200–300 mL of water (this contained a drop

Table I. Primers used for the genus-specific PCR and *C. imicola* specific PCR.

Primer	Sequence
PanCulF	5'-GTAGGTGAACCTGCGGAAGG-3
PanCulR	5'-TGCGGTCTTCATCGACCCAT-3'
Cul-Imicola	5'-ATTACAGTGGCTTCGGCAAG-3'

of detergent to reduce surface tension) was placed at the base of each trap. The collected insects were transported to the laboratory in the water-filled collecting beaker and then recovered and preserved in 90% ethanol. *Ceratopogonidae* were first separated from all other insects. Identification of *Culicoides* was initially based upon wing pattern, and confirmed subsequently by mounting a number of specimens on microscope slides [14, 27].

2.2. Extraction of genomic DNA

Single specimens of *Culicoides* were used for the extraction of DNA. After grinding of the insect in a mortar and pestle containing liquid nitrogen, the genomic DNA was extracted with the DNeasy Tissue kit (Qiagen, USA) according to the manufacturer's instructions.

2.3. PCR primers

The gene analysis software Vector NTI (Informax Inc, USA) was used to compare the ITS1 rDNA sequences of the 11 species of *Culicoides* retrieved from Genbank/EMBL databases for the design of primers. Two sets of primers were defined. The first set of primers (PanCulF/PanCulR) was designed for a genus-specific PCR, the other set of primers (Cul-imicola/PanCulR) for a *C. imicola* specific PCR. Table I lists the sequences of the primers used.

2.4. PCR amplification of ITS1

The rDNA locus consists of one very long array of repeat units (500–700 repeats per genome). Each repeat is 9 kb long and consists of the genes 18S, 5.8S, and 28S rDNA. These genes are separated by spacers, the internal transcribed spacers (ITS1 and ITS2), and the intergenic spacer (IGS) (Fig. 1). The ITS1 of each of the eight most abundant species of *Culicoides* and of *C. imicola* was amplified by genus-specific PCR using the PanCulF/PanCulR primers and by *C. imicola* specific PCR using the Cul-imicola/PanCulR primers. Reactions for the PanCulF/PanCulR primers were performed in a total volume of 50 µL consisting of 10 × PCR reaction buffer, 1.5 mM $MgCl_2$, 250 µM of each dATP, dCTP, dGTP and dTTP (Eurobio, France), 200 ng of each primer, and 2.5 U Taq DNA polymerase under the following cycling conditions: an initial denaturation stage at 94 °C for 5 min; then 30 cycles at 94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min and a final extension phase at 72 °C for 10 min. The reaction for the Cul-imicola/PanCulR primers was the same as described above except for the hybridation temperature that was increased to 60 °C. A volume of 1 µL of genomic DNA was added to each PCR reaction and samples without DNA were included in each amplification run to exclude carry-over contamination. PCR products were examined by electrophoresis in a 1.0% agarose gel with ethidium bromide.

3. RESULTS

3.1. Collection of *Culicoides* along coastal mainland France

The genus *Culicoides* was represented by 16 197 specimens belonging to 44 species, and were collected during 109 nights of sampling between April and November 2002. No specimens of *C. imicola* were found. Table II summarises the *Culicoides* species composition, expressed as a percentage of the total *Culicoides* population sampled. The species mentioned in this table are the ones representing more than 1% of the whole population.

Table II. Number and percentage of adult *Culicoides* captured between April and November 2002 along coastal mainland France (19 sites/8 months/109 light trap collections).

No.	*Culicoides*	Number collected	% of the total population sampled
1	*C. newsteadi*	11 898	73.5
2	*C. obsoletus*	1 290	8.0
3	*C. scoticus*	877	5.4
4	*C. circumscriptus*	526	3.2
5	*C. griseidorsum*	430	2.7
6	*C. pulicaris*	188	1.2
7	*C. lupicaris*	178	1.1
8	*C. submaritimus*	173	1.1

Table III. Genbank and NCBI accession numbers and lengths of the ITS1 sequences (base pairs) of 11 species of *Culicoides*.

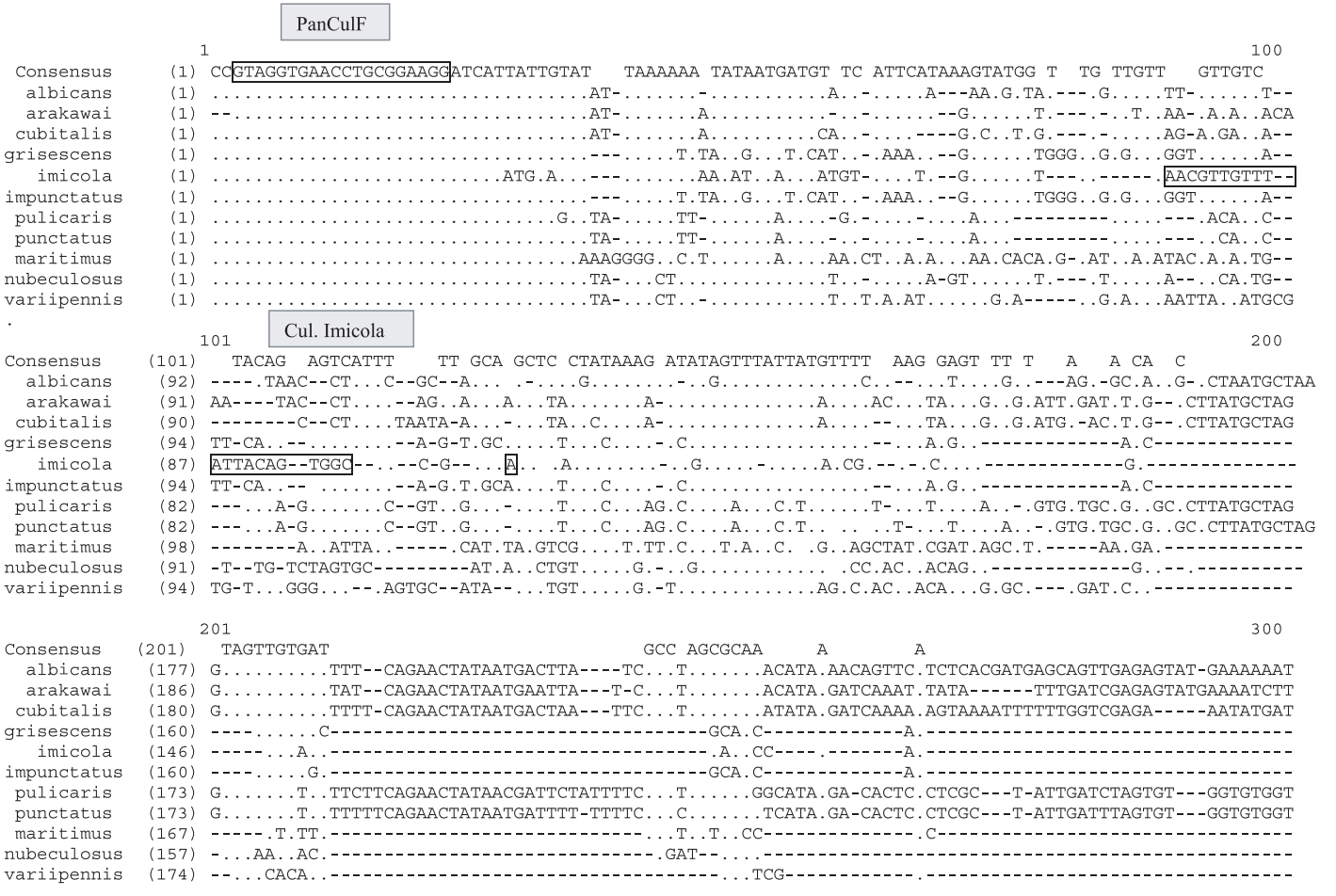
Culicoides	Accession number	Length of ITS1 sequence (base pairs)
C. albicans	AJ417980	461
C. arakawai	AJ489503	460
C. cubitalis (= *C. kibunensis*)	AJ417979	457
C. grisescens	AJ417987	346
C. imicola	AF074019	316
C. impunctatus	AJ417986	330
C. maritimus	AJ417981	427
C. nubeculosus	AJ417982	332
C. pulicaris	AJ417983	469
C. punctatus	AJ417984	463
C. variipennis	U48380	347

3.2. Alignment of the ITS1rDNA of the 11 species

The ITS1 of the 11 species of *Culicoides* retrieved from EMBL/Genbank databases were aligned using the gene analysis software Vector NTI. Table III lists the accession numbers of each of these sequences and their length. The alignment of the ITS1 sequences (the alignment length was 590 bp including the gaps) revealed two highly conserved regions, between 2 and 28 bp and 564 and 591 bp, respectively (Fig. 2). These regions were used for the design of the genus specific primers but could also be useful in the future for the molecular identification of *Culicoides,* for studying their molecular genetics and epidemiology, and for vector systematics. The positions of the primers selected for the molecular detection of *Culicoides* are shown in grey-coloured boxes (Fig. 2).

3.3. Amplification of ITS1 by genus-specific PCR

PCR reactions using genus-specific primers (PanCulF/PanCulR) were performed on the eight most-abundant species captured along the French Mediterranean coastline


PanCulF
Cul. Imicola

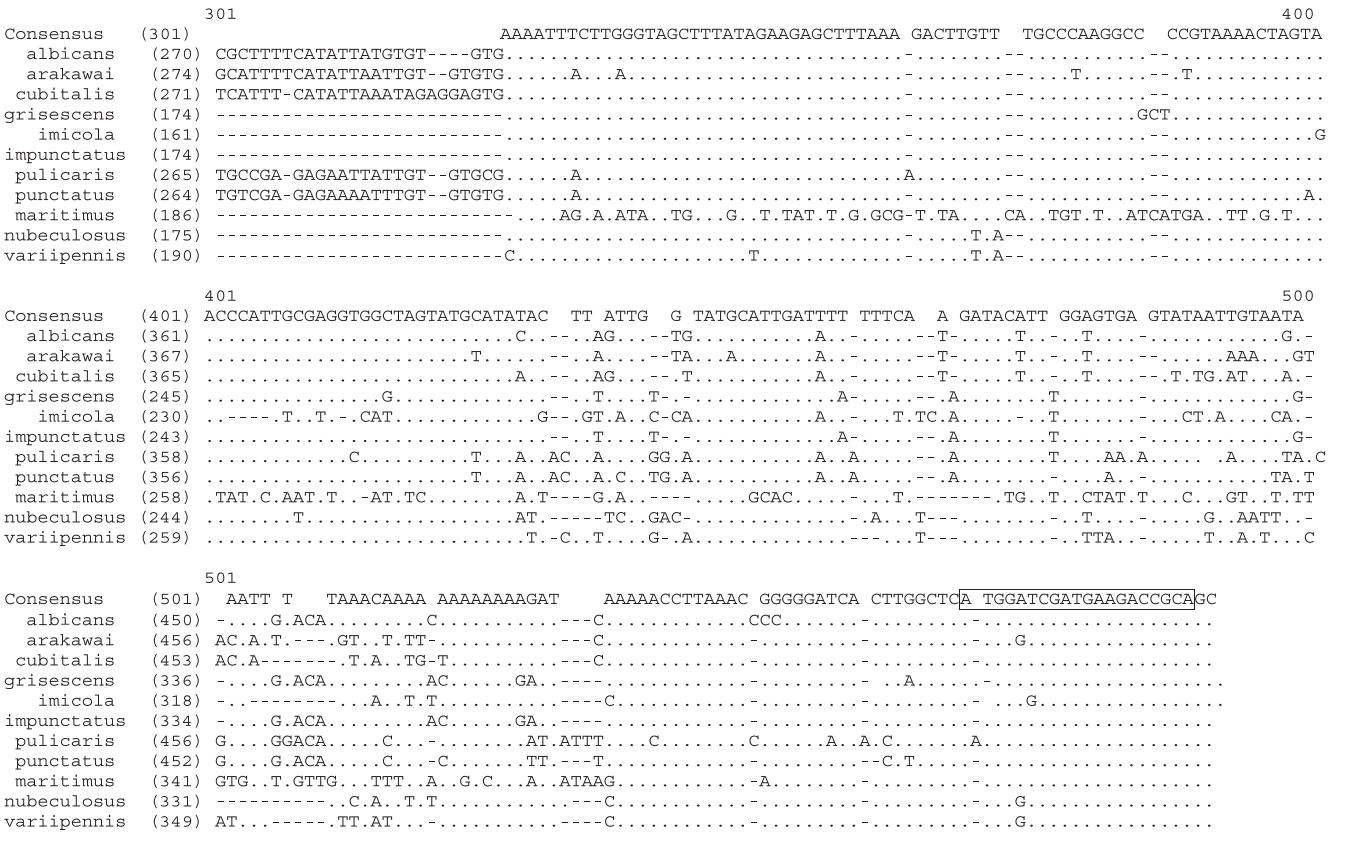


Figure 2. Alignment of the ITS1 of the 11 species of *Culicoides* known to date. In the figures, dots correspond to the conserved nucleotides. Boxed-letters indicate the sequences used to design the primers used for genus-specific PCR and the *C. imicola*-specific PCR. Block letters are the nucleotides that differ between species.

PanCulR

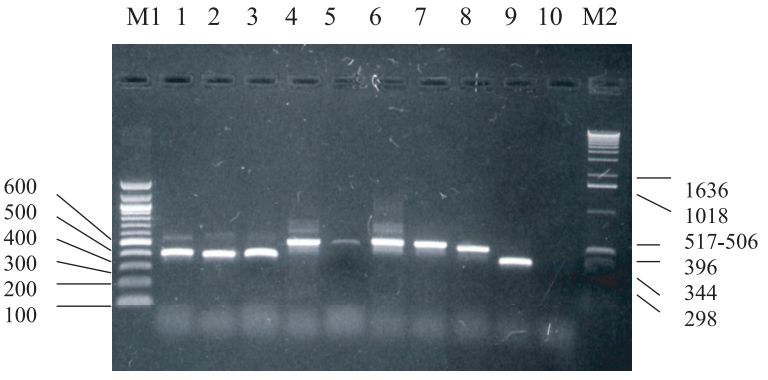


Figure 3. Genus amplification of the 9 *Culicoides* species ITS1 by PCR (PanCulF/PanCulR). M1 represents 100 bp DNA ladder, M2 marker X (Roche, USA). Lanes 1 to 10 correspond respectively to *C. newsteadi, C. obsoletus, C. scoticus, C. circumscriptus, C. griseidorsum, C . pulicaris, C. lupicaris, C. submaritimus, C. imicola*, Negative Control.

and reference *C. imicola* specimens collected in Corsica in 2001, 150 km distant from southern France. Agarose gel electrophoresis (Fig. 3) showed that the genus-specific amplified PCR products of the nine *Culicoides* species tested (*C. newsteadi, C. obsoletus, C. scoticus, C. circumscriptus, C. griseidorsum, C. pulicaris, C. lupicaris, C. submaritimus* and *C. imicola*) occurred between 316 and 500 bp (when compared against the 100 bp DNA ladder marker).

As expected, no band was detected in the negative control. The PCR was also performed on other species of *Culicoides* less commonly found on the French Mediterranean mainland (*C. sahariensis, C. kibunensis, C. maritimus, C. punctatus*, etc.). The results confirmed that the sequences targeted by our primers are highly conserved (data not shown).

3.4. Molecular identification of *C. imicola* by specific ITS1 PCR

PCR reactions were performed in order to evaluate the specificity of *C. imicola* primers (Cul-imicola/PanCulR) for *C. imicola,* compared to other *Culicoides* species. Agarose gel electrophoresis (Fig. 4) showed no specific PCR products for each of the eight non-*C. imicola* ITS1 species tested (*C. newsteadi, C. obsoletus, C. scoticus, C. circumscriptus, C. griseidorsum, C. pulicaris, C. lupicaris,* and *C. submaritimus*). However, a specific band (303 bp) was amplified for *C. imicola*. Again, no band was detected in the negative control. Thus, we developed a specific tool for the detection of *C. imicola.*

3.5. Specificity of the PCR assay

Because surveillance activities for most vector species are performed using pooled genus specimens, we wished to evaluate whether our specific PCR assay was useful to detect *C. imicola* within a pool of other biting midges. Agarose gel electrophoresis (Fig. 5) showed that no specific PCR products were amplified in lane 1, corresponding to a pool of one specimen of each of the eight most-abundant *Culicoides* species (*C. newsteadi, C. obsoletus, C. scoticus, C. circumscriptus, C. griseidorsum, C. pulicaris, C. lupicaris, C. submaritimus*). The addition of one or ten specimens of *C. imicola* (lanes 2 and 3 respectively) to this pool yielded the expected band of 303 bp following PCR amplification. Again, no band was

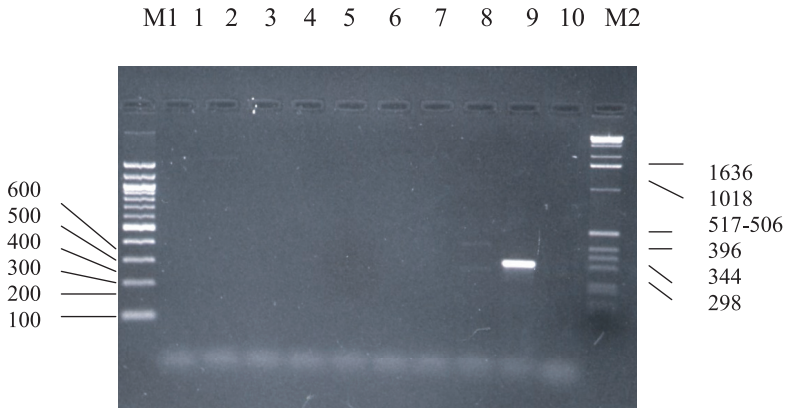


Figure 4. Amplification of the 9 *Culicoides* species ITS1 by specific PCR (Cul-Imicola/PanCulR). M1 represents 100 bp DNA ladder, M2 marker X (Roche, USA). Lanes 1 to 10 correspond respectively to *C. newsteadi, C. obsoletus, C. scoticus, C. circumscriptus, C. griseidorsum, C. pulicaris, C. lupicaris, C. submaritimus, C. imicola,* Negative Control.

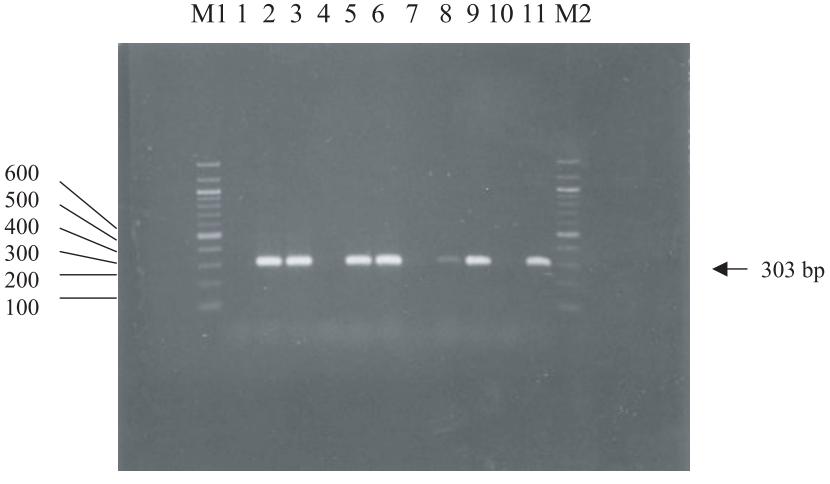


Figure 5. The specificity of the *Culicoides imicola* specific PCR amplification (Cul-Imicola/PanCulR). M1 and M2 represent 100 bp DNA ladder. Lane 1 corresponds to a mix of 1 specimen of each of the eight most abundant species *C. newsteadi, C. obsoletus, C. scoticus, C. circumscriptus, C. griseidorsum, C. pulicaris, C. lupicaris, C. submaritimus.* Lanes 2 and 3 are equivalent to lane 1 with one and 10 specimens of *C. imicola* respectively. Lane 4 corresponds to ten specimens of *Forcipomyia* spp. Lanes 5 and 6 correspond to lane 4 with one and 10 specimens of *C. imicola* respectively. Lane 7 corresponds to a pool of different Diptera families (Psychodidae, Chironomidae, Culicidae, Cecidomyidae, Sciaridae, Tipulidae) and other orders of insects (*Lepidoptera, Coleoptera, Hymenoptera, Homoptera*) recovered from a light-trap. Lanes 8 and 9 correspond to lane 7 plus one and 10 specimens of *C. imicola* respectively. Lane 10 is the negative control. Lane 11 is a *C. imicola* positive control.

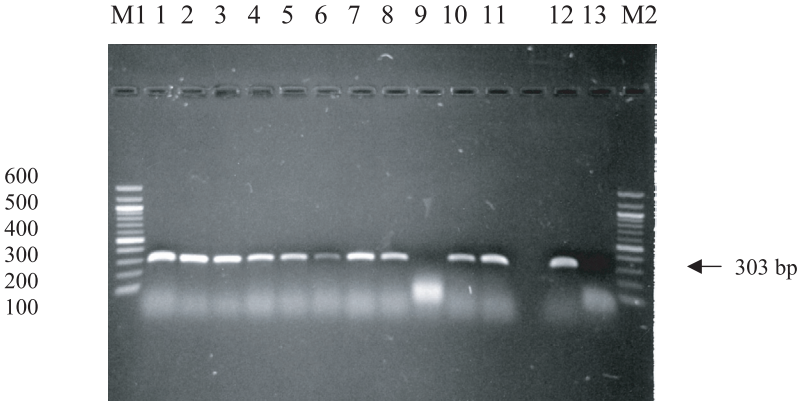


Figure 6. Sensitivity of the *Culicoides imicola* specific PCR amplification (PanCulR/Cul-Imicola). M1 and M2 represent 100 bp DNA ladder. Lanes 1 to 8 correspond respectively to one specimen of *C. imicola* in 25, 50, 100, 200, 400, 800, 1 600 and 3 200 specimens of *C. newsteadi.* Lane 9 corresponds to 6.5 mg of dried materials recovered from a light trap without any *Culicoides* specimens. Lanes 10 and 11 correspond to lane 9 with one and 10 specimens of *C. imicola* respectively. Lane 11 corresponds to lane 9 with ten specimens of *C. imicola*. Lanes 12 and 13 correspond respectively to the positive and negative controls.

detected (lane 4) following PCR on a pool of 10 specimens of *Forcipomyia* spp., a genus belonging to the Ceratopogonidae family. However, a specific band was amplified when one or ten specimens of *C. imicola* (lanes 5 and 6 respectively) were added to the pool. Finally, a mixture of different insect families recovered from a light-trap equivalent to 6.5 mg of dried materials (lane 7) did not result in the amplification of specific DNA, but when one or ten specimens of *C. imicola* were added (lanes 8 and 9, respectively), amplification of a specific band resulted.

3.6. Sensitivity of the PCR assay

In order to assess the sensitivity of the technique, *C. imicola*-specific PCR reactions were performed on *C. imicola* within a range of pooled specimen sizes. Figure 6 shows agarose gel electrophoresis of specific PCR products that were amplified in serial samples containing one specimen of *C. imicola* in 25, 50, 100, 200, 400, 800, 1 600 and 3 200 specimens of *C. newsteadi* (lanes 1 to 8 respectively). Lane 9 corresponds to 6.5 mg of dried material recovered from a light trap without any *Culicoides* specimens, showing that no band was detected. Finally, when one or ten specimens of *C. imicola* were added to the dried material pool from the trap, a specific band was amplified (lanes 10 and 11). As expected, no band was detected in the negative control (lane 13).

4. DISCUSSION

In its recent re-emergence in the Mediterranean Basin, and particularly in Corsica in 2000 and 2001, it is believed that BTV was transmitted principally by the classical Afro-Asiatic insect vector *C. imicola*. However, in the eastern Mediterranean Basin, outbreaks of the disease have occurred in Bulgaria, Serbia, Kosovo, Croatia, Montenegro, northern Greece and in Bosnia Herzegovina where *C. imicola* has not been detected during insect surveys. Recently, the virus was isolated from *Culicoides* represented by one or more species of the

C. obsoletus complex on mainland Italy [24] and *C. pulicaris* on the island of Sicily [5].

C. imicola is also the vector for the African horse sickness, another orbiviral disease that can be especially devastating to equids. The monitoring of *Culicoides* species is therefore essential in areas at risk for the management of animal health. Since the biology of *C. imicola* and its persistence in a given biotope appear to be largely influenced by climatic conditions, the spectre of possible global climate change suggests that monitoring efforts should be sustained over a long period of time. This is particularly true in areas like southern France where trapping frequencies will be increased to sample high quantities of insects for an early detection of *C. imicola*. However, in such extensive monitoring programmes the morphological identification of *Culicoides* has been limited to the labour-intensive and time consuming techniques of morphological taxonomy, species prevalence and geographic distribution. This is the reason why we decided to investigate the possibility to detect *Culicoides* spp. and *C. imicola* using molecular tools. From the molecular point of view, recent studies on this genus hold promise for the development of tools that will lead to the rapid and reliable identification of *C. imicola* and other potential vectors of BTV [15]. The nuclear ribosomal DNA arrays of eukaryotic organisms usually consist of tandemly repeated copies of the transcriptional unit and non-transcribed spacer, the rDNA transcriptional unit consisting of three gene regions (i.e. 18S, 5.8S and 28S) separated by transcribed spacers [8]. As shown in Figure 1, the external transcribed spacer (ETS) is located upstream of the 18S gene, with the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) located between either genes 18S and 5.8S, or between 5.8S and 28S, respectively. The rDNA has several unique features which make it an ideal target for use in DNA species-diagnostic assays [6]. It contains regions of varying evolutionary rates, from highly conserved regions (e.g. 5.8S and 18S rRNA genes) to more variable ones (e.g. spacers). Species from other genera have been studied also showing that rDNA provides genetic markers for the identification of mosquitoes (*Anopheles gambiae, An. nili and An. minimus* using ITS) [9, 10, 13, 22, 26]. In another study the ITS1 sequences of *C. arakawai*, and eight other species, were used to produce an initial partial phylogeny of the species within the genus [15].

Thus, our objective in this study was to establish a diagnostic test for the rapid detection of the main BTV-transmitting species, *C. imicola*. Even if the ITS1 sequences of most of the species present in the Mediterranean Basin remain unknown, the choice of the primers in a highly conserved region amongst sequenced species, should serve for the detection of the majority of *Culicoides* species. After genus PCR reaction, the nine *Culicoides* species (including *C. imicola*), yielded an amplified fragment of 316 to 500 bp. Other *Culicoides* species could also be detected by these primers (data not shown), thus demonstrating the validity of our initial hypothesis. Interestingly, the highly variable size of the amplified products (up to 180 bases) illustrates that ITS-1 may differ widely between species of the same genus. This reinforces the potential interest of ITS-1 for phylogenetic studies of *Culicoides*. We are now sequencing all our PCR products with the objective of performing a phylogenetic analysis.

The specific *C. imicola* primer was designed to amplify in a more variable region where numerous substitutions or deletions were present. The primer that was selected proved to be specific for *C. imicola*. Because two forward primers combined with a unique reverse primer provides resolution at the genus and species levels, development of a useful multiplex PCR should be possible. We are now evaluating this possibility as well as developing a real-time PCR test as a faster and quantitative diagnostic tool.

When we evaluated the sensitivity of our PCR, we found that only one specimen of each *Culicoides* was sufficient to obtain a

PCR product. In addition, our PCR was able to specifically detect one *C. imicola* within 3 200 other *Culicoides* (Fig. 6) or within 6.5 mg of dried materials collected from a light trap. That amount of material (6.5 mg) represents approximately a third of the insect mass collected by a light trap during the maximum *Culicoides* activity period in France. Interestingly, the sensitivity of our PCR was greatly improved when the genomic DNA was diluted by 10 before amplification. The robust nature of this technique for *C. imicola* surveillance is also demonstrated by its ability to specifically detect one specimen of *C. imicola* within 8 other *Culicoides* species, within 10 specimens of *Forcipomyia* spp., or within a pool of different Diptera families and other orders of insects.

Another potential application of this work will be to adapt this technique to *Culicoides* larvae, in order to help in the identification of the larvae and then in the characterisation of their morphological differences and also to define the larvae biotope of *C. imicola* and the biological parameters which are involved in their development (duration, diapause, aestivation).

In conclusion, we developed a rapid and qualitative diagnostic tool for *Culicoides* spp. and *C. imicola* that should be useful for routine epidemiological and surveillance monitoring of this orbivirus vector. Our work also suggests that specific forward primers for other *Culicoides* species that are vectors for bluetongue or African horse sickness virus could be designed and used as well.

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