Journal of Medical Entomology, XX(XX), 2022, 1–6
https://doi.org/10.1093/jme/tjac065
Research

ENTOMOLOGICAL SOCIETY OF AMERICA
SHARING INSECT SCIENCE GLOBALLY

OXFORD

Molecular Biology/Genomics

Rapid and Efficient Detection by PCR of *Culicoides insignis* (Diptera: Ceratopogonidae), the Main Vector of Bluetongue Virus (BTV) in the Neotropical Region

Mahia Mariel Ayala,[1,3] Florentina Díaz,[1] María Victoria Micieli,[1] Gustavo Ricardo Spinelli,[2] and María Marcela Ronderos[1]

[1]Centro de Estudios Parasitológicos y de Vectores (CEPAVE) CONICET-UNLP, Boulevard 120 S/N entre Avda 60 y calle 64, (1900) La Plata, Buenos Aires, Argentina, [2]Instituto de Limnología "Dr. Raúl A. Ringuelet" (ILPLA) CONICET-UNLP Boulevard 120 y 62, Número 1437, (1900) La Plata, Buenos Aires, Argentina, and [3]Corresponding author, e-mail: ayalamahia@cepave.edu.ar

Subject Editor: Maria Anice Sallum

Received 25 January 2022; Editorial decision 20 April 2022.

Abstract

Bluetongue virus (BTV) causes a viral, non-contagious disease that mainly affects sheep, cattle, and wild and farmed ruminants causing damage to these animals and significant economic losses. *Culicoides insignis* Lutz, the major BTV vector in South America, is one of the most abundant species in Argentina and commonly associated with cattle farms. The morphological identification of *Culicoides* spp. is routinely carried out with the aid of morphological keys, which mainly refer to the wing patterns, sensillae of palpi, and antennal flagella. Molecular tools applied to taxonomy provide a rapid and efficient method of identification of vector species. An easy protocol for the extraction of total DNA from single midges is herein described, and a forward primer for rapid and reliably test detection by polymerase chain reaction of *C. insignis* is developed.

Key words: biting midges, primers, molecular detection

Ceratopogonidae are one of the common fly families in the Neotropical Region from coastal habitats to high mountains (Borkent and Spinelli 2007). Adult females of the biting midge genus *Culicoides* Latreille are haematophagous. They are notorious pest that cause nuisance to livestock and humans, and are commonly known as "biting midges," "no-see-ums," or "punkies" in English speaking countries, "polvorines," "manta blanca," "chaquistes," or "jejenes" (which also may refer to Simuliidae) in Spanish speaking countries, and "mosquito pólvora" or "maruim" in Brazil (Spinelli and Ronderos 2005, Díaz et al. 2018).

Many species of biting midges are efficient vectors of arboviruses, protozoa, and filarial nematodes (Wirth and Blanton 1959). One of the most important diseases transmitted by *Culicoides* is caused by the Bluetongue virus (BTV). It is a non-contagious disease that mainly affects sheep, cattle, and wild and farmed ruminants, causing serious damage to these animals and significant economic losses (Mellor et al. 2000). In the New World, BTV spread is limited to ca. 35 S and 40 N (MacLachlan 2011).

The Bluetongue disease is well known in Central America and the Caribbean, and even in the Lesser Antilles (Legisa et al. 2014). Most of the information available for this region is due to the efforts of the Regional Bluetongue team since the early 1980s (Greiner et al. 1992, Thompson et al. 1992) and the Interamerican Bluetongue team (Legisa et al. 2014). These teams performed a large surveillance in eleven countries in the region, including serologic detections in cattle, sheep and goats, and virus isolations. Regarding the vector species suspected to be responsible for BTV transmission among ruminants, Walton and Osburn (1992), Greiner et al. (1990), and Mo et al. (1994) isolated BTV from *C. insignis* Lutz, *C. filarifer* Hoffman, and *C. pusillus* Lutz. This suggests that these three species (mainly *C. insignis*) were the primary species in viral transmission in this region (Tanya et al. 1992, Legisa et al. 2014). *Culicoides insignis* is one of the most frequent and abundant species found in Southeastern United States, the Caribbean Basin, and Central and South America, which is primarily associated with cattle farms (Ronderos et al. 2003, Ayala et al. 2018).

© The Author(s) 2022. Published by Oxford University Press on behalf of Entomological Society of America. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com.

Downloaded from https://academic.oup.com/jme/advance-article/doi/10.1093/jme/tjac065/6604338 by guest on 08 June 2022

In South America, this virus has been isolated in Brazil, Argentina, Peru, Ecuador, and Guyana. In June 2013, an outbreak of BTV was confirmed in a herd of 99 dairy sheep of the Lecauna breed in the State of Rio de Janeiro, Brazil. This resulted in the death of four pregnant sheep's and six that were affected, 16 male lambs became ill, and 2 died (Legisa et al. 2014). In the Brazilian Paraná state, Scolari et al. (2010) detected high seroprevalences in cattle and sheep. These seroprevalences, among virus isolations and the favorable weather conditions for the vector life cycle, suggest that BTV could spread to areas and countries surrounding that region, including Paraguay, Uruguay, and northeastern Argentina, where BTV has been detected and isolated (Legisa et al. 2014) although this disease has never been reported.

Accurate identification of biting midges is essential for the understanding of disease epidemiology and vector control. Morphologically, this task is routinely carried out with the aid of dicotomic keys, species descriptions (including original descriptions), and comparisons with previously identified specimens housed in reliable entomological collections. The most important morphological characters used by entomologists include wing patterns, the presence or absence of sensillae on antennal flagella, the presence and shapes of palpal pits on the 3rd segment of maxillary palpi, numbers of mandibular teeth, and male and female genitalia. Conversely, the molecular tools applied to taxonomy provide a rapid and efficient method to the identification of vector species. However, because of their small size, traditional methods of DNA extraction are not frequently used in *Culicoides*, and it is usual to use commercial kits, which are expensive, especially in underdeveloped countries. The development of species-specific primers complements the identification process of midges. The region of ITS-1 of the ADNr has shown a great potential in order to perform a reliable polymerase chain reaction (PCR)-based procedure (Cêtre-Sossah et al. 2004, Stephan et al. 2009). Herein, we describe an easy protocol for the extraction of total DNA from single midges and to develop a forward primer for the rapid and reliable detection of *C. insignis*, the main vector of BTV in the Neotropics.

Materials and Methods

The entomological survey was carried out in SW Misiones province, Argentina (27° 25′ 59.70″ S; 55° 53′ 35.67″ W) from January to December 2013.

Culicoides were captured with CDC light traps into collecting jars containing 70% ethanol. Collected specimens were extracted and preserved in properly labeled tubes with 70% ethanol. In the laboratory, specimens of *Culicoides* were dissected and slide-mounted in Canada balsam for microscopic examination using the techniques by Borkent and Spinelli (2007). They were identified using various keys (Wirth and Blanton 1959, Wirth and Blanton 1973, Spinelli et al. 2005), descriptions, and the Atlas of wing photographs of Neotropical species (Wirth et al. 1988). They were also compared with slide-mounted specimens in the Entomology Division of the Museo de La Plata, AR (MLPA).

Morphologically, *Culicoides insignis* is placed in the *Culicoides guttatus* group of the subgenus *Hoffmania*, reviewed by Spinelli et al. (1993). The females are easily differed from congeners of this species group by a combination of three wing characters: the r-m crossvein is distinctly dark, the vein R_3 is dark up to the point where it turns abruptly forward to meet the costa, and by the single distal pale spot in cell M_1; other useful characters are the third palpal segment bearing an irregular sensory pit and the distal sensilla coeloconica always present on flagellomeres 1, 3, 5, 7, and 9–13, and sometimes present on flagellomeres 2, 4, 6, and 8. The male wing frequently exhibits a second faint pale spot at wing margin in cell M_1.

Extraction of Genomic DNA

The thorax and abdomen of specimens identified morphologically as *C. insignis* were used for the extraction of DNA.

The genomic DNA analysis was performed by the following instructions: preserved samples were homogenized in 160 µl of extraction buffer (Tris/HCl 100 mM, EDTA 50 mM y ClNa 0.1 M), 20 µl of SDS 2%, 40 µl of CTAB 2%, and 1 µl of Proteinase K. After incubating for 1 h at 65°C, 200 µl of chloroform: isoamyl alcohol (24:1 v/v) was added. Homogenized material was centrifuged at 12,000 rpm for 5 min, after recovering the supernatant and was added to 150 µl of 3M KAc. After centrifugation for 5 min at 12,000 rpm, the supernatant was recovered and transferred to a new tube and 1000 µl of cold absolute ethanol were added to precipitate DNA. It was then centrifuged for 5 min at 12,000 rpm and the resulting pellet was washed with 800 µl ethanol 70%. After centrifugation for 5 min at 12,000 rpm, the pellet was allowed to dry at room temperature and resuspended in 30 µl of double-distilled water (ddH_2O). DNA concentrations were determined through absorbance at 260 nm on a spectrophotometer. The figures were edited with Paint Net software V4.3.2 Version.

PCR Primers Design and PCR Amplification by Species-Specific Primers

The ITS-1 region of *C. insignis* was amplified by a specific PCR for *Culicoides*, using the PanCulF/PanCulR primers (Cêtre-Sossah et al. 2004). Reactions were performed in a total volume of 20 µl, consisting of 1x PCR reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 10 pmol of each primer, and 0.5 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. These amplification products were sequenced.

The reaction for the Cul_insig F/PanCulR primers was as the one described above except for the hybridation temperature that was 51°C. Sequences of the primers used during this study are shown in Table 1. Samples without DNA were included as negative controls, and PCR products were examined by electrophoresis in a 1.0% agarose gel with ethidium bromide.

We use BioEdit Version 7.2.5 software (Hall 1999) to align attained forward and reverse DNA sequences obtained for *C.*

Table 1. Primers used for the genus-specific PCR (Pan Cul F and R) and the species-specific primer for *C. insignis* Lutz

Primers	Sequence 5′ to 3	Tm (°C)	References
PanCul F	GTAGGTGAACCTGCGGAAGG	54	Cêtre-Sossah et al., 2004
PanCul R	TGCGGTCTTCATCGACCCAT	58	Cêtre-Sossah et al., 2004
Cul_insig F	GCTATTTGATTAGCAGCT TTGC	51	Present study

Downloaded from https://academic.oup.com/jme/advance-article/doi/10.1093/jme/tjac065/6604338 by guest on 08 June 2022

insignis specimens. Each base of the sequences was compared one by one with the reading of its corresponding electropherogram, and it was verified that both were coincident.

With the sense and antisense sequences corrected, a consensus sequence was generated, which was used as a query to verify the match with the amplified region and the organism in question. We worked with the National Center for Biotechnology Information - NCBI NR database (non-redundant) (NCBI Resource Coordinators 2016), using the BLAST tool [National Center for Biotechnology Information, Basic Local Alignment Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=Blast Search&LINK_LOC=blasthome), Madden 2013], where the alignment was carried out according to the parameters established by default of the program.

The consensus sequence was used to design the primer. We used the online software Muscle-Multiple Sequence Alignment [EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/muscle/), Integrated DNA Technologies (https://www.idtdna.com/pages/tools/oligoanalyzer), Edgar 2004] to identify the species-specific region of *C. insignis* and also to compare the ITS-1 sequences of *C. insignis* and ITS-1 sequences of other species of extraneotropical *Culicoides* obtained from the GenBank (https://www.ncbi.nlm.nih.gov/genbank/): *C. variipennis* (Coquillett)—GenBank KP310092; *C. imicola* Kieffer—GenBank JN408479; *C. albicans* (Winnertz)—GenBank AJ417980; *C. obsoletus* (Meigen)—GenBank AY861152; all of these species are considered BTV vectors, except *C. albicans*.

Once the primer was manually designed, the online program Oligoanalyzer 3.0 was used to evaluate autohybridization and/or possible dimer formation with the antisense primer and the temperature at which base pairing occurs.

The primer specificity was checked through alignment with *Culicoides* sequences published in GenBank with the aid of the online BLAST tool.

Results

Collection of *Culicoides*

During the fieldwork, 3,590 specimens of *Culicoides* were collected. Of these, 2,952 were identified as *C. insignis*, representing the 93.4% of the total collected specimens.

DNA Extraction Protocol

To standardize a DNA extraction protocol, we evaluated several modifications of the protocol performed by Doyle and Doyle (1987). The midge body parts were treated differently, obtaining the best results with the following procedures:

1. The addition of SDS 2% and Proteinase K to the lysis buffer for the cell lysis and protein denaturation.
2. The combination of solvents and salts: in the first extraction, we use chloroform: isoamyl alcohol (C:IAA) (24:1 v/v), latter we add K Acetate (KAc), for the DNA purification and protein removal.
3. The use of cold ethanol absolut and ethanol 70%, with no incubation, and the resuspension of DNA in ddH_2O.

DNA extracted (Fig. 1) by this methodology usually yields sufficient DNA for several rounds of PCR amplifications.

Bioinformatic Analysis of the ITS-1 Sequences of *C. insignis* and Other Species of Non-neotropical *Culicoides*

The alignment reveals the highly conserved regions between other species of *Culicoides*, and the species-specific region of *C. insignis*.

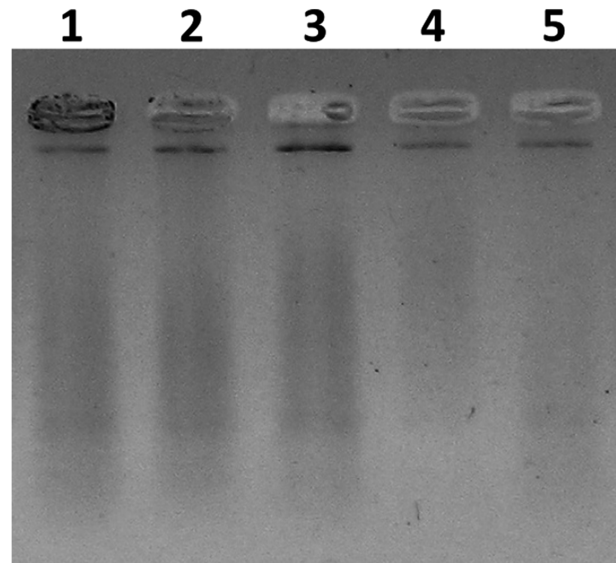

Fig. 1. Genomic DNA from individual specimens of *C. insignis* Lutz. Lanes: 1 and 2—whole individual especimen; 3 to 5—thorax and abdomen of the individual specimens of *C. insignis*.

The alignment length between the sequence ITS-1 of *C. insignis* and other analyzed sequences was 565 bp, including the gaps. This alignment revealed several highly conserved regions; the larger ones are among between 323 and 337 bp and 339 and 356 bp, respectively (Fig. 2). A *C. insignis*-specific region was identified in the region between bases 79 and 107. This being the region used for the design of the specie specific primer.

The obtained ITS-1 sequence specific for *C. insignis* was submitted to GenBank with an appropriate accession number (MH566998).

PCR Optimization for Identification of *C. insignis* Using Molecular Tools

The species-specific *C. insignis* forward primer (Cul_insigF) was designed to be used in a PCR with the PanCul reverse primer (PanCulR).

Initially, to test the specificity of the primer in vitro, we worked with the five most-abundant species captured during the fieldwork (*C. debilipalpis* Lutz; *C. limai* Barretto, *C. paraensis* (Goeldi); *C. lahillei* (Iches); and *C. venezuelensis* Ortiz & Mirsa). The agarose gel electrophoresis showed a specific PCR product for *C. insignis* at 300 bp, and no cross reactions with other *Culicoides* species; also no band was detected in the negative control (Fig. 3).

Later, because of the lack of ITS-1 sequences in the species of the guttatus group or some sequence of any neotropical *Culicoides* in the database, the primer in sílico specificity was checked through alignment with other *Culicoides* sequences published in GenBank with the aid of the online BLAST tool, where the alignment was carried out according to the parameters established by default of the program.

DNA quantification by spectrophotometer indicated that each PCR reaction carried out contained between 7.3 and 8.1 ng.

Discussion

Specific identification of *Culicoides* has been carried out until recently on the basis of morphological characters. Likewise, the molecular biology techniques, such as the PCR and automatic sequencing

Downloaded from https://academic.oup.com/jme/advance-article/doi/10.1093/jme/tjac065/6604338 by guest on 08 June 2022

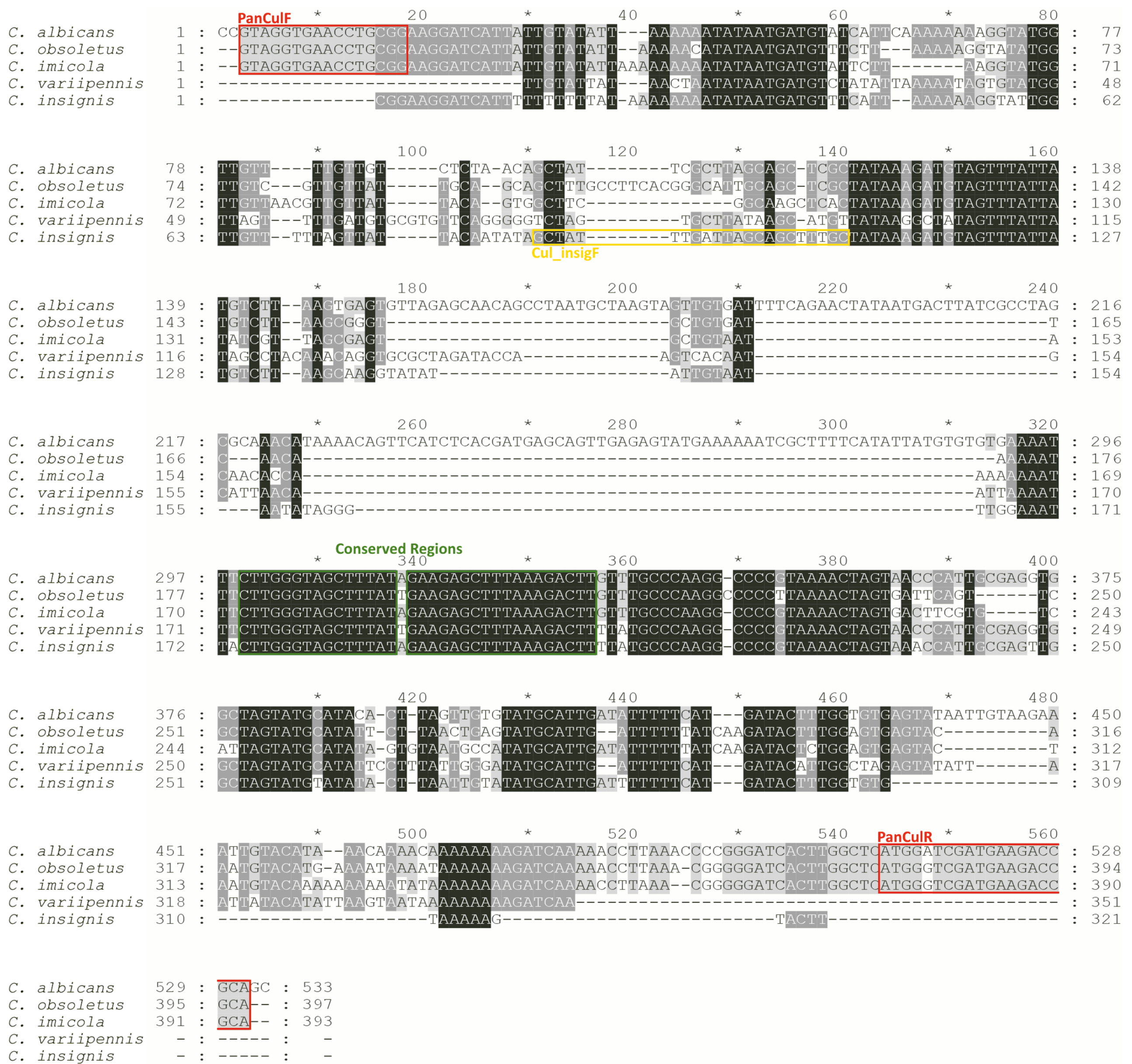

Fig. 2. Alignment of the ITS-1 of the 5 species of *Culicoides* Latreille. Boxt-letters show the hybridization site of the PanCul F- Pan Cul R primers; and the location of the Cul_insigF primer. The conserved regions among the analyzed species are painted in black.

applied to the study of the insects considered vectors of diseases, have become an important step in the resolution of taxonomic conflicts.

Several protocols have been published for the extraction of DNA in other families of Diptera (Gaillard and Strauss 1990, Golczer and Arrivillaga 2008, Rosero et al. 2010). Due to the small size of *Culicoides* spp., traditional methods for individual DNA extractions have not been used within this genus. Although the use of commercial kits is simpler, they may be quite expensive in developing countries. For this reason, we herein describe an easy protocol for the extraction of total DNA from single specimens, without the use of RNases or commercially available DNA isolation kits, even for the DNA precipitation, the overnight incubation was not necessary. The DNA extracted by this methodology is enough for several PCR amplifications.

Previous studies showed that the ITS region allowed the detection by PCR of other BTV vector species, like *C. imicola* Kieffer (Cêtre-Sossah et al. 2004) and *C. dewulfi* Goetghebuer (Stephan et al. 2009). We developed a primer for the rapid and reliable detection of *C. insignis*, the main vector of BTV in Neotropical region.

GenBank has 46,428 sequences of the genus *Culicoides*: 5,520 COI sequences and 129 sequences of ITS-1 and 156 of ITS-2. Most of these sequences come from species that are not found in the Neotropical region. Augot et al. (2017) provide a sequence D1D2 for *C. paraensis* (Goeldi) when they carried out a phylogenetic analysis of 42 *Culicoides* species from Europe, America, and Africa, using *mtDNA* COI and the D1 and D2 regions of the 28S rDNA. Despite the sanitary importance as a vector for BTV in the Neotropics, the information available about sequences of nucleotide of *C. insignis* is very limited. In addition to our ITS-1 sequence (MH566998), recently Rios et al. (2021) submitted the first sequences of the COI gene from this species from Brazil (MT806183, MT806184, MW871560, MW871561, and MW871562).

Downloaded from https://academic.oup.com/jme/advance-article/doi/10.1093/jme/tjac065/6604338 by guest on 08 June 2022

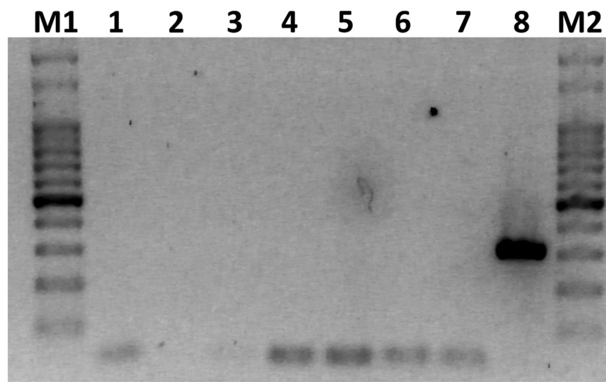

Fig. 3. PCR amplification by species-specific primers (Cul_insig F/PanCulR). Lanes: 1—Negative control; 2—*C. debilipalpis* Lutz; 3—*C. limai* Barretto, 4—*C. paraensis* (Goeldi); 5—*C. lahillei* (Iches); 6—*C. venezuelensis* Ortiz & Mirsa; 7—*C. spp*; 8—*C. insignis* Lutz. M1 and M2 represent 100bp DNA ladder.

There are a few sequences of 28S ribosomal RNA gene and COI gene of two species included in the guttatus group, *C. batesi* Wirth & Blanton (KF286361, KF286362 and KY707787) and *C. guttatus* (Coquillett) (KY707785 and KY707785), but there is not any available sequence of ITS region (ITS-1 or ITS-2) of other species in this group. Unfortunately, since they are different regions of the genome, they cannot be compared with our ITS-1 sequence of *C. insignis*.

The guttatus species group is included in the subgenus *Hoffmania* Fox. Three species of *Culicoides* (*Hoffmania*) from the Palearctic region that do not belong to the guttatus group, have sequences of ITS-1 region, are as follows:

Culicoides (*Hoffmania*) *indianus* Macfie (MH809964, MH809963, and MH809962), *C.* (*H.*) *peregrinus* Kieffer (AB462274), and *C.* (*H.*) *sumatrae* Macfie (AB462276). The specificity of the primer Cul_insig F was checked through alignment with these sequences of *Culicoides* species included in the subgenus *Hoffmania*, using the online BLAST tool. The results showed that the specific region of *C. insignis* is not found in any of these species, so this primer would not match with any other genomic sequence in the ITS region belonging to another species of the genus.

The use of molecular biology tools applied to the specific identification of species will contribute significantly in cryptic species (Garros et al. 2014). We designed a forward primer since a specific sequence of the ITS-1 region of *C. insignis*, which does not generate amplification products in the other analyzed *Culicoides* species captured during the fieldwork (Fig. 3), thereby simplifying the taxonomic identification process of *Culicoides* midges. With a specificity and sensitivity of 100%, this method could also be used for larval identification and epidemiological surveillance of these species.

Acknowledgments

We thank Dr. Darío Balcazar of Centro de Estudios Parasitológicos y de Vectores (CEPAVE) for the assistance on the edition of figures. We also acknowledge to anonymous reviewers for their appropriate comments on the manuscript. This study was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

Funding

Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

Conflicts of Interest

The authors declare no conflict of interest. The funder had no role in all the steps involved in the development of this work, such as the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References Cited

Augot, D., B. Mathieu, L. Hadj-Henni, V. Barriel, S. Zapata Mena, S. Smolis, D. Slama, F. J. Randrianambinintsoa, G. Trueba, M. Kaltenbach, *et al*. **2017**. Molecular phylogeny of 42 species of *Culicoides* (Diptera, Ceratopogonidae) from three continents. *Parasite*. 24: 23.

Ayala, M. M., V. Le Gall, D. Martí, L. Walantus, and G. R. Spinelli. **2018**. Population fluctuation of *Culicoides insignis* Lutz (Diptera: Ceratopogonidae) in Posadas, Misiones, Argentina. *Rev. Soc. Entomol. Argent*. 77: 16–20.

Borkent, A., and G. R. Spinelli. **2007**. Neotropical Ceratopogonidae (Diptera: Insecta). In J. Adis, J. R. Arias, G. Rueda-Delgado, and K. M. Wantzen (eds.), *Aquatic biodiversity in Latin America (ABLA)*, Pensoft, Sofia-Moscow. 4: 1–200.

Cêtre-Sossah, C., T. Baldet, J. Delecolle, B. Mathieu, A. Perrin, C. Grillet, and E. Albina. **2004**. Molecular detection of *Culicoides* spp. and *Culicoides imicola*, the principal vector of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. *Vet. Res*. 35: 325–337.

Díaz, F., C. Mangudo, G. R. Spinelli, R. Gleiser, and M. M. Ronderos. **2018**. The immatures of *Culicoides trilineatus* (Diptera: Ceratopogonidae) potential vector of the Bluetongue virus. *J. Med. Entomol*. 55: 877–883.

Doyle, J. J., and J. L. Doyle. **1987**. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull*. 19: 11–15.

Edgar, R. C. **2004**. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 32: 1792–1797.

Gaillard, C., and F. Strauss. **1990**. Ethanol precipitation of DNA with linear polyacrylamide as carrier. *Nucleic Acids Res*. 18: 10.

Garros, C., T. Balenghien, S. Carpenter, J. Delecolle, R. Meiswinkel, A. Pedarrieu, I. Rakotoarivoni, L. Gardes, N. Golding, J. Barber, *et al*. **2014**. Towards the PCR-based identification of palearctic Culicoides biting midges (Diptera: Ceratopogonidae): results from an international ring trial targeting for species in the subgenus *Avaritia*. *Parasit. Vectors*. 7: 1–9.

Golczer, G., and J. Arrivillaga. **2008**. Modification of a standard protocol for DNA extraction from smaller sandflies (Phlebotominae: *Lutzomyia*). *Rev. Colomb. Entomol*. 34: 199–202.

Greiner, E. C., W. I. Knausenberger, M. Mesersmith, W. L. Kramer, and E. P. J. Gibbs. **1990**. *Culicoides* spp. (Diptera: Ceratopogonidae) associated with cattle in St. Croix, Virgin Islands, and their relevance to bluetongue viruses. *J. Med. Entomol*. 27: 1071–1073.

Greiner, E.C., C.L. Mo, V. Tanya, L.H. Thompson, M.T. Oviedo, and T.E. Walton. **1992**. Interamerican Bluetongue team. Vector ecology of bluetongue viruses in Central America and the Caribbean. In: Walton TE and Osburn BI (eds.), *Proceedings of the Second International Symposium on Bluetongue, African Horse Sickness, and Related Orbiviruses,* Paris. CRC Press, Boca Raton, FL. **1992**; 320-324.

Hall, T. A. **1999**. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser*. 41: 95–98.

Legisa, D. M., F. N. Gonzalez, and M. J. Dus Santos. **2014**. Bluetongue virus in South America, Central America and the Caribbean. *Virus Res*. 182: 87–94.

MacLachlan, J. N. **2011**. Bluetongue: history, global epidemiology, and pathogenesis. *Prev. Vet. Med*. 102: 107–111.

Madden, T. **2013**. The BLAST Sequence Analysis Tool. In: *The NCBI Handbook* [Internet]. 2nd edition. National Center for Biotechnology Information (US), Bethesda, MD. Available from: https://www.ncbi.nlm.nih.gov/books/NBK153387/

Mellor, P. S., J. Boorman, and M. Baylis. **2000**. *Culicoides* biting midges: their role as arbovirus vectors. *Annu. Rev. Entomol*. 45: 307–340.

Downloaded from https://academic.oup.com/jme/advance-article/doi/10.1093/jme/tjac065/6604338 by guest on 08 June 2022

Mo, C. L., L. H. Thompson, E. J. Homan, M. T. Oviedo, E. C. Greiner, J. González, and M. R. Sáenz. **1994**. Bluetongue virus isolations from vectors and ruminants in Central America and the Caribbean. *Am. J. Vet. Res*. 55: 211–215.

NCBI Resource Coordinators. **2016**. Database resources of the National Center for Biotechnology Information. *Nucl. Acids Res*. 44(D1):D7–D19.

Rios, R. R. S., M. C. A. Santarém, K. A. L. Ribeiro Júnior, B. A. de Melo, S. G. M. da Silva, N. C. da Silva, V. R. V. dos Santos, J. M. dos Santos, A. E. G. Santana, and A. B. Fraga. **2021**. *Culicoides insignis* Lutz. 1913 (Diptera: Ceratopogonidae) biting midges in Northeast of Brazil. *Insects*. 12: 366.

Ronderos, M. M., G. R. Spinelli, I. Lager, and F. Díaz. **2003**. La importancia sanitaria de los jejenes del Género Culicoides (Diptera: Ceratopogonidae) en la Argentina. . *Universidade Gama Filho. Entomología y Vectores*. 10: 601–612.

Rosero, D. A., L. A. Gutierrez, A. V. Cienfuegos, L. M. Jaramillos, and M. Correa. **2010**. Optimization of a DNA extraction procedure for anopheline mosquitoes. *Rev. Colomb. Entomol*. 36: 260–263.

Scolari, A.P.R., R.R.T.B. Richartz, P. Prichla, R.R. Pagnoncelli, C.S. Sotomayor, and R.D. Ollhoff. **2010**. *Seroprevalence of Bluetongue virus in domestic ruminants of three regions in the State of Paraná—Southern Brazil*. World Buiatrics Congress, Santiago de Chile, Chile.

Spinelli, G. R., E. C. Greiner, and W. W. Wirth. **1993**. The Neotropical bloodsucking midges of *Culicoides guttatus* group of the subgenus Hoffmania (Diptera: Ceratopogonidae) . *Contrib. Am. Entomol. Inst*. 27: 1–91.

Spinelli, G.R., and M.M. Ronderos. **2005**. Ceratopogonidae (Formas hematófagas). In O. D. Salomón (eds.), *Artrópodos de interés medico en Argentina, Serie enfermedades transmisibles*. Publicación monográfica 6, Buenos Aires, Argentina. 6: 61–66.

Spinelli, G. R., M. M. Ronderos, F. Díaz, and P. Marino. **2005**. The bloodsucking biting midges of Argentina (Diptera: Ceratopogonidae) . *Mem. Inst. Oswaldo Cruz*. 100: 137–150.

Stephan, A., P. H. Clausen, B. Bauer, and S. Steuber. **2009**. PCR identification of *Culicoides dewulfi* midges (Diptera: Ceratopogonidae), potential vectors of bluetongue in Germany. *Parasitol. Res*. 105: 367–371.

Tanya, V. N., E. C. Greiner, and E. P. J. Gibbs. **1992**. Evaluation of *Culicoides insignis* (Diptera: Ceratopogonidae) as a vector of bluetongue virus. *Vet. Microbiol*. 32: 1–14.

Thompson, L.H., C.L. Mo, M.T. Oviedo, and E.J. Homan. **1992**. Inter-American Bluetongue team. Prevalence and incidence of Bluetongue viruses in the Caribbean Basin: serologic and virologic findings. In: Walton TE and Osburn BI (eds.), *Proceedings of the Second International Symposium on Bluetongue, African Horse Sickness, and Related Orbiviruses*. Paris. CRC Press, Boca Raton, FL. 106–113.

Walton, T., and B. Osburn. **1992**. Bluetongue, African Horse sickness and related orbiviruses. In: Walton TE and Osburn BI (eds.), *Proceedings of the Second International Symposium on Bluetongue, African Horse Sickness, and Related Orbiviruses. Paris*. CRC Press, Boca Raton, FL. 1042 pp.

Wirth, W. W., and F. S. Blanton. **1959**. Biting midges of the genus *Culicoides* from Panamá (Diptera: Heleidae) *Proc. U.S. Nat. Mus*. 109: 237–482.

Wirth, W. W., and F. S. Blanton. **1973**. A review of the maruins of biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) in the Amazon basin. *Amazoniana*. 4: 405–470.

Wirth, W. W., A. L. Dyce, and G. R. Spinelli. **1988**. An atlas of wing photographs, with a summary of the numerical characters of the Neotropical species of *Culicoides* (Diptera: Ceratopogonidae). *Contrib. Am. Entomol. Inst*. 25: 1–72.

Downloaded from https://academic.oup.com/jme/advance-article/doi/10.1093/jme/tjac065/6604338 by guest on 08 June 2022