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Development and preliminary evaluation of a real-time polymerase chain reaction for the identification of *Culicoides obsoletus sensu strictu*, *C. scoticus* and *C. montanus* in the Obsoletus Complex in Italy

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Summary

A real-time polymerase chain reaction (PCR) using a green fluorescence dye, followed by a comparison of derivative melting curves in the post-amplification phase, was developed to distinguish species of *Culicoides* within the Obsoletus Complex. The selected target sequence was internal transcribed spacer 2 (ITS 2) of the ribosomal DNA (rDNA). Using the newly developed method, 140 midges were morphologically classified in the Obsoletus Complex and were processed. The results were compared to those obtained by combining the morphological identification with the gel based reverse transcriptase (RT)-PCR. By analysing the species-specific pattern of the dissociation curves, it was possible to identify 52 midges as *Culicoides scoticus*, 82 midges as *C. obsoletus sensu strictu* and 6 as *C. montanus*. These results matched those obtained by the combination of gel-based PCR and morphological identification used on a routine basis. Given its diagnostic flexibility, rapid results, automation capability, high quality result performance and expression, the real-time ITS 2 rDNA PCR appears to be more functional and efficient than the gel-based PCR, especially when dealing with large-scale monitoring of midges belonging to the Obsoletus Complex.

Keywords

Bluetongue, *Culicoides*, *Culicoides montanus*, *Culicoides obsoletus*, *Culicoides scoticus*, Italy, PCR, Polymerase chain reaction, Obsoletus complex, Virus.

Introduction

Bluetongue (BT) is an infectious, vector-borne non-contagious viral disease which affects wild and domestic ruminants. It is caused by the BT virus (BTV), a double-stranded RNA virus that is a member of the genus *Orbivirus* (7), family *Reoviridae*. To date, 24 distinct serotypes have been identified (9). Midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are the biological vectors. Bluetongue has incurred severe economic losses to the European livestock industries over the past 10 years. As vectors, *Culicoides* play an essential role in determining the presence and spread of the virus worldwide (10). In Europe, depending on different latitudes, several species of *Culicoides* have been involved in these unprecedented series of outbreaks. In countries of southern Europe, although midges of the Pulicaris (2) and Obsoletus Complexes (5, 19) might have had an important role, *Culicoides imicola* Kieffer, 1913, is still the most important vector. Conversely, species of the

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Obsoletus Complex, together with *C. dewulfi* Goetghebuer, 1936 and *C. chiopterus* Meigen, 1830 (8, 17) are thought to be the main vectors of BTV in countries of northern Europe.

In Italy, the Obsoletus Complex includes at least three species, as follows:

- *C. obsoletus* Meigen, 1818
- *C. scoticus* Downes and Kettle, 1952
- *C. montanus* Schakirzjanova, 1962.

Their identification, which is based on morphology only, is very difficult, and consequently they are usually considered as a 'complex' (16).

Following the first bluetongue outbreaks in Italy in 2000, a BT National Surveillance Plan (NSP) was implemented by the Italian Ministry of Health (14) and included entomological activities. Knowing the species of *Culicoides* implicated in an outbreak is crucial for predicting and understanding the evolution of the infection. Entomological surveillance is based on weekly catches of *Culicoides* in about 200 permanent sites across Italy, in accordance with standardised methods for the collection and identification of midges (11). To integrate and improve the morphological identification and to differentiate *C. obsoletus sensu strictu* (*ss*), *C. scoticus* and *C. montanus* at species level, a gel-based polymerase chain reaction (PCR) is currently for molecular identification through in Italy (12, 13).

The combination of morphological and bio-molecular identification systems, although enhancing accuracy, is a complex procedure that is not appropriate for entomological surveys which routinely process very large quantities of samples. In such condition a real-time PCR (rPCR) would be more efficient. In comparison to the gel-based PCR, the rPCR has enormous potential. Its diagnostic flexibility, speed, low risk of contamination and objective interpretability make this test procedure more functional and considerably quicker than the conventional PCR. Furthermore, since the amplified target is detected through the PCR cycles, the process can be automated with ease and the lack of manipulation of the amplified products for detection considerably reduces the risk of contamination which often creates a further source of doubt when interpreting results in conventional PCR systems, especially when large numbers of samples are processed daily. This study describes the implementation of a gel-based PCR that targets the internal transcribed spacer 2 (ITS 2) of the ribosomal DNA for the identification of *C. obsoletus ss*, *C. scoticus* and *C. montanus* in the Obsoletus Complex into an rPCR.

Materials and methods

Insects

Twenty-five *Culicoides*, 10 *C. obsoletus ss* males, 10 *C. scoticus* males and 5 *C. montanus* females, collected within the NSP, were selected to set up the assay and construct the control plasmids. The midges were morphologically and genetically identified at species level, according to Campbell and Pelham-Clinton (1) and Delécolle (6), comparing their DNA sequences with those published in Genbank.

DNA extraction and species-specific real-time polymerase chain reaction

Total DNA was extracted from single midges using the automated Maxwell 16 system (Promega, Madison, Wisconsin) with the DNA IQ casework sample kit, according to manufacturer's instructions The DNA was eluted into 20 µl of DNase-free water.

A multiplex rPCR targeting the segment of the rDNA ITS 2 was developed. DNA from individual midges was amplified using the primers listed in Table I (13). The reaction volume was 25 µl, consisting of 12.50 µl of Power SYBR green (Applied Biosystems, Carlsbad, California), 1 µM of 5.8SF and MON227R primers, 0.2 µM (each) of 28SR, SCOT194R and MOU316F primer and 5 µl of DNA. The PCR was performed using the 7900 HT Fast Real Time PCR system (Applied Biosystems).

The thermal profile was set at 95°C for 10 min to activate the polymerase, followed by an initial denaturation step at 95°C for 30 sec, and then 40 cycles at 95°C for 30 sec, 58°C for

Table I
Primer pairs used and their amplification pattern

Target	Primer pairs	Amplicon (bp)
ITS 2	5.8 SF-28 SR	400
Culicoides montanus	5.8 SF-MON227R	252
C. scoticus	5.8 SF-SCOT194R	213
C. obsoletus ss, *C. montanus*	MOU316F-28 SR	89

ITS2 internal transcribed spacer 2

30 sec, 72°C for 30 sec. After the PCR was completed, a melting curve analysis was conducted with a denaturising phase from 60°C to 95°C according to the standard ramp rate. The values of the peak T_m are presented as indicated by the SDS 2.3 software (Applied Biosystems).

Positive clones

To minimise the false-negative results due to a lack of amplification and to create PCR-positive controls, the species-specific target sequences of *C. obsoletus ss*, *C. scoticus* and *C. montanus* were cloned into bacterial plasmids.

For each species, the entire ITS 2 target was amplified and the purified products were sequenced with the external primers described. The sequencing reaction was set up using the Big Dye Terminator kit (Applied Biosystems), the excess of dyes was removed using the Big Dye XTerminator Purification kit (Applied Biosystems) and the nucleotide sequences were determined with the DNA sequencer ABI PRISM 3100 (Applied Biosystems). Rough sequence data were assembled using Contig Express (Vector NTI suite 9.1, Invitrogen, Carlsbad) and consensus sequences were aligned and compared to homologous sequences available on Genbank, namely: AY599796, AY599811 (*C. scoticus*), AY599779, AY599769 (*C. montanus*), AY599780, AY599795 (*C. obsoletus ss*) with ClustalX (21).

The purified PCR products were cloned into plasmid PCR4 Topo (Invitrogen, Carlsbad) by chemically competent *Escherichia coli*. To assess the lack of mutations induced by the cloning procedure, positive clones were selected by restriction analysis and then sequenced as previously described. Transformed bacteria were stored at –70°C in glycerol and included as positive reaction controls in each run.

Field study

To asses the robustness of the PCR, 140 randomly selected adult female midges belonging to the Obsoletus Complex were identified using the traditional gel-based PCR (13). A second PCR targeting the ITS 1 (3), the new developed real-time assay and results were compared.

Results

Species-specific real-time polymerase chain reaction and positive clones

The amplicons of the three species were successfully cloned and sequenced. When aligned with the homologous sequences published in Genbank, the sequences obtained in this study confirmed that either the cloning procedure or the orientation within the plasmids were correct (Fig. 1).

The target region of the 25 midges was amplified by rPCR. The derivative melting curve plot obtained in the presence of Power SYBR green produced clearly distinguishable melting peaks, one of 73.1°C (standard deviation [SD] ± 0.2) for *C. obsoletus ss*, one of 79.2°C (SD ± 0.2) for *C. scoticus* and two peaks of 72.4°C (SD ± 0.2) and 79.2°C (SD ±0.4) for *C. montanus* (Fig. 2).

Field trial

When the 140 female midges were examined using the gel-based PCR routinely used in the IZS A&M entomological laboratory or the second PCR targeting ITS 1 and the newly developed rPCR, the results concurred. In

particular, the three systems identified 52 midges as *C. scoticus*, 82 as *C. obsoletus ss* and 6 midges as *C. montanus*. The mean T_m value of *C. scoticus* midges was 79.3°C (SD ± 0.5), of *C. obsoletus ss* it was 73.2°C (SD ± 0.5) and for *C. montanus* midges, a first peak with a T_m value of 72.8°C (SD ± 0.2) and a second peak of 79.2°C (SD ± 0.2) were recorded.

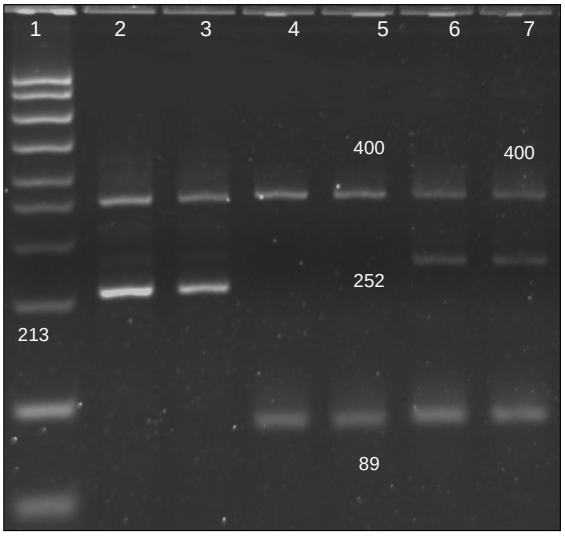

Figure 1
Amplification pattern of the species-specific plasmids
Lane 1 DNA ladder 50-2 000 bp (Biorad, Hercules, California)
Lanes 2-3 Plasmid *C. scoticus*
Lanes 4-5 Plasmid *C. obsoletus ss*
Lane 6-7 Plasmid *C. montanus*
The numbers give the length of the amplicons

Discussion

As in other diagnostic fields, molecular technology is now appearing more and more frequently in the entomological sector. Numerous molecular procedures targeting different genome segments of the *Culicoides* species are now available for identification. The techniques most commonly used are those for ITS 2 rDNA (13) or ITS 1 rDNA (3, 15, 20) or mitochondrial cytochrome oxidase subunit I (COI) sequences (18). They are mainly used to confirm conventional morphological identification when it is not possible to distinguish species of *Culicoides* based on morphology alone. This is particularly true for species belonging to the Obsoletus Complex where females of member species, such as *C. obsoletus ss*, *C. scoticus* and *C. montanus* are extremely difficult to distinguish using classical morphology procedures.

Identifying these species and determining their distribution have recently become particularly critical from an epidemiological viewpoint. Indeed, species of the Obsoletus Complex are widespread across Europe and some of these appear to be involved in BTV transmission in northern Europe.

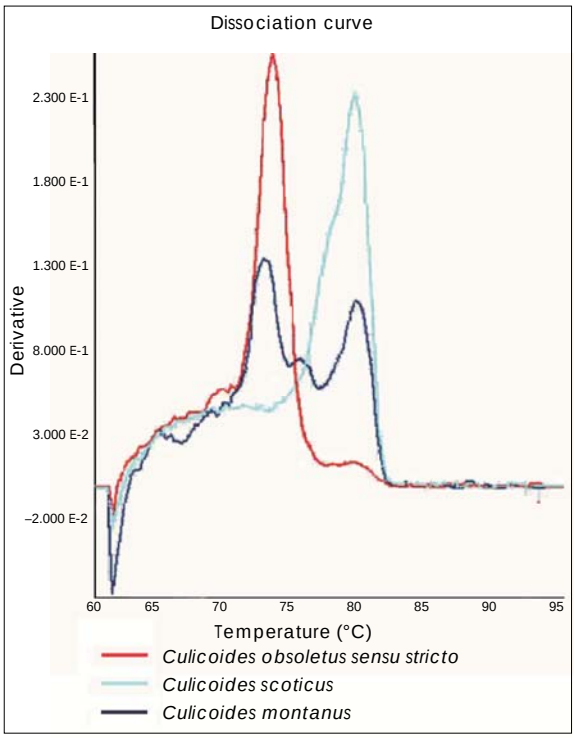

Figure 2
Analysis of amplicons obtained by testing the ITS 2 rDNA of *Culicoides obsoletus ss*, *C. scoticus* and *C. montanus* from plasmids
Real-time polymerase chain reaction was performed using the Power SYBR green as dye and the 7900 HT (Applied Biosystems, Carsberg, California)
Following real-time polymerase chain reaction amplification of the template, the products were analysed for the temperature at which the double-stranded DNA dissociates
This dissociation curve analysis revealed a species-specific pattern

To solve the Obsoletus Complex identification problem, a gel-based PCR targeting ITS 2 rDNA (13) was integrated in conventional morphological identification procedures (12).

Although improving the diagnostic efficacy of conventional morphological identification, the

gel-based PCR, still however, reveals certain weaknesses which could be serious disadvantages in a scenario such as the NSP where thousands of midges need to be identified rapidly. In such circumstances, an rPCR would be much more functional. To date, only one rPCR has been developed and has been used with success for *C. imicola* identification (4). In this study, an rPCR targeting ITS 2 rDNA was developed. The rPCR was able to correctly identify species of *C. obsoletus ss*, *C. scoticus* and *C. montanus*. The melting peaks were clear and well defined for plasmids and for male and female samples. Although in field samples a slight increase in SD was reported which was probably due to different amounts of DNA used in the reactions, the mean temperatures were distinct and no there was overlapping between the occurrence of species-specific peaks. With an automated method of DNA extraction, the newly developed system was faster than the gel-based PCR. The automation of the extraction phase also improved the quality of the product and satisfactory levels of DNA purity were obtained from a whole midge. The purity of the DNA is important as it can interfere with the subsequent amplification phase (F. Monaco, unpublished data). In each run, plasmids which enclosed species-specific targets of the amplification, were included. This enabled monitoring of the amplification process to avoid false reactions due to PCR inhibition phenomena and, if successfully amplified, to provide a 'reference' peak to interpret the results for each identified species. Another advantage of the new method is that it could be applied to parts (i.e. legs) of midges (data not shown) without losing any diagnostic efficacy. This meant that the remaining parts, such as the wings and body, could be preserved for further morphological and/or virological investigations. The possibility of performing both molecular identification and virus isolation on large quantities of midges would be of great value when assessing vector competency.

Conclusions

On the basis of these results, the real-time ITS 2 rDNA fluorogenic PCR method developed in this study was capable of correctly identifying midges belonging to *C. obsoletus ss*, *C. scoticus* and *C. montanus*, with 100% agreement with the gel-based PCR. Although additional testing is required to test the robustness of the method, diagnostic flexibility, rapidity of results, automation capability, higher quality result performance and expression of the real time ITS 2 rDNA PCR means that it is more functional and efficient than the gel-based PCR, especially when dealing with large-scale monitoring of midges belonging to the Obsoletus Complex.

References

1. Campbell J.A. & Pelham-Clinton E.C. 1960. A taxonomic review of the British species *Culicoides* Latreille (Diptera, Ceratopogonidae). *Proc R Soc Edinb (B)*, **67**, 181-302.
2. Caracappa S., Torina A., Guercio A., Vitale F., Calabrò A., Purpari G., Ferratelli V., Vitale M. & Mellor P.S. 2003. Identification of a novel bluetongue virus vector species of *Culicoides* in Sicily. *Vet Rec*, **153**, 71-74.
3. Cetre-Sossah C., Baldet T., Delécolle J.-C., Mathieu B., Perrin A., Grillet C. & Albina E. 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.
4. Cetre-Sossah C., Mathieu B., Setier-Rio M.L., Grillet C., Baldet T., Delécolle J.-C. & Albina E. 2008. Development and evaluation of a real-time PCR assay for *Culicoides imicola*, one of the main vectors of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. *Res Vet Sci*, **85** (2), 372-382.
5. De Liberato C., Scavia G., Lorenzetti R., Scaramozzino P., Amaddeo D., Cardati G., Scicluna M., Ferrari G. & Autorino G.L. 2005. Identification of *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a vector of bluetongue virus in central Italy. *Vet Rec*, **156**, 301-304.
6. Delécolle J.-C. 1985. Nouvelle contribution à 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 des sciences de la vie et de la terre, Strasbourg, 238 pp (bluetongue.cirad.fr/FichiersComplementaires/1985_Delecolle_TheseOriginaleCulicoides.pdf accessed on 1 June 2010).

7. de Mattos C.A., de Mattos C.C., Osburn B.I. & MacLachlan N.J. 1994. Evolution of the L2 gene of strains of bluetongue virus serotype 10 isolated in California. *Virology*, **201**, 173-177.
8. Dijkstra E., van der Ven I.J.K., Hölzel D.R., Van Rijn P.A. & Meiswinkel R. 2008. *Culicoides chiopterus* as a potential vector of bluetongue virus in Europe. *Vet Rec*, **162**, 422.
9. Erasmus B.J. 1990. Bluetongue. *In* Virus infection of ruminants, Vol. 3 (Z. Dinter & B. Morein, eds). Elsevier Science, Amsterdam, 227-237.
10. Gibbs E.P. & Greiner E.C. 1994. The epidemiology of bluetongue. *Comp Immunol Microbiol Infect Dis*, **17**, 207-220.
11. Goffredo M. & Meiswinkel R. 2004. Entomological surveillance of bluetongue in Italy: methods of capture, catch analysis and identification of *Culicoides* biting midges. *In* Bluetongue, Part I. Proceedings Third International Symposium, Taormina, 26-29 October 2003 (N.J. MacLachlan & J.E. Pearson, eds). *Vet Ital*, **40**, 260-265.
12. Goffredo M., Benedetto L., Di Marcello V. & Monaco F. 2008. Identification of *C. obsoletus*, *C. scoticus*, *C. montanus* and *C. dewulfi*: an integrated morphological and genetic approach. *In* Proc. Bluetongue Satellite Symposium Bluetongue in Europe: back to the future!, 7 June, Brescia. Epizone, Brescia, 81.
13. Gomulski L.M., Meiswinkel R., Delécolle J.-C., Goffredo M. & Gasperi G. 2005. Phylogenetic relationships of the subgenus *Avaritia* Fox, 1955 including *Culicoides obsoletus* (Diptera, Ceratopogonidae) in Italy based on internal transcribed spacer 2 ribosomal DNA sequences. *Syst Entomol*, **30**, 619-631.
14. Italian Ministry of Health 2000. Nota del 6 ottobre 2000 n. 600.6/24461/82N/3903 Sistema Nazionale di Sorveglianza per Bluetongue. Italian Ministry of Health, Rome.
15. Mathieu B., Perrin A., Baldet T., Delécolle J.-C., Albina E. & Cetre-Sossah C. 2007. Molecular identification of Western European species of obsoletus complex (Diptera: Ceratopogonidae) by an internal transcribed spacer-1 rDNA multiplex polymerase chain reaction assay. *J Med Entomol*, **44** (6), 1019-1025.
16. Meiswinkel R., Gomulski L.M., Delécolle J.-C., Goffredo M. & Gasperi G. 2004. The taxonomy of *Culicoides* vector complexes – unfinished business. *In* Bluetongue, Part I. Proceedings Third International Symposium, Taormina, 26-29 October 2003 (N.J. MacLachlan & J.E. Pearson, eds). *Vet Ital*, **40**, 151-159.
17. Meiswinkel R., Van Rijn P., Leijs P. & Goffredo M. 2007. Potential new *Culicoides* vector of bluetongue virus in northern Europe. *Vet Rec*, **161**, 564-565.
18. Nolan D.V., Carpenter S., Barber J., Mellor P.S., Dallas J.F., Mordue A.J. & Piertney S.B. 2007. Rapid diagnostic assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. *Vet Microbiol*, **124**, 82-94.
19. Savini G., Goffredo M., Monaco F., Di Gennaro A., Cafiero M.A., Baldi L., De Santis P., Meiswinkel R. & Caporale V. 2005. Bluetongue virus isolations from midges belonging to the Obsoletus Complex (*Culicoides*, Diptera: Ceratopogonidae) in Italy. *Vet Rec*, **157**, 133-143.
20. Stephan A., Clausen P.H., Bauer B. & Steuber S. 2009. PCR identification of *Culicoides dewulfi* midges (Diptera: Ceratopogonidae), potential vectors of bluetongue in Germany. *Parasitol Res*, **105**, 367-371
21. Thompson J.D., Gibson T.J., Plewiak F., Jeanmougin F. & Higgins D.G. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*, **25**, 4876-4882.

