Elsevier Editorial System(tm) for Veterinary Parasitology
Manuscript Draft

Manuscript Number: Vetpar-D-11-4677R1

Title: Molecular characterisation of Swiss Ceratopogonidae (Diptera) and evaluation of real-time PCR assays for the identification of Culicoides biting midges

Article Type: Research Paper

Keywords: Culicoides, vector identification, barcode, real-time PCR, specificity, cryptic species.

Corresponding Author: Professor Alexander Mathis,

Corresponding Author's Institution: University of Zurich

First Author: Claudia E Wenk

Order of Authors: Claudia E Wenk; Christian Kaufmann; Francis Schaffner; Alexander Mathis

Abstract: Biting midges of the genus Culicoides (Diptera, Ceratopogonidae) are vectors of several viruses of veterinary relevance, and they can cause insect bite hypersensitivity. As the morphological identification of these tiny insects is a difficult task in many cases, alternative approaches are expedient. With the aim to develop real-time PCRs, we determined partial mitochondrial cytochrome oxidase I gene (mt COI) sequences from 380 Culicoides midges representing three regions of Switzerland, namely the Alps, Midland north of the Alps (Atlantic climate), and South of the Alps (Mediterranean climate). The same region was also sequenced from non-biting midges of the genera Atrichopogon, Brachypogon, Dasyhelea, Forcipomyia and Serromyia. A total of 21 Culicoides species were identified by morphology. Sequence variability (haplotypes) was observed in all species. For each of C. grisescens and C. obsoletus, a novel cryptic species was identified. Whereas all individuals of C. grisescens and of the cryptic C. obsoletus species (O2) originated only from Alpine sites, the known C. obsoletus (O1) species was found in all three regions. Further, a sister taxon to C. pulicaris was identified based on the mt COI sequences and named Culicoides sp. Alignments of available mtCOI sequences from Ceratopogonidae (GenBank, this study) were used to design real-time PCR primers and probes to distinguish C. chiopterus, C. deltus, C. dewulfi, C. grisescens (including the cryptic species), C. imicola, C. lupicaris, C. obsoletus O1, C. obsoletus O2, C. pulicaris, C. scoticus and Culicoides sp. Specificities of primers and probes was tested with cloned targets representing 1 to 4 haplotypes of 18 Culicoides spp. and 1 haplotype each from 4 other Ceratopogonidae. No cross-reactivity was observed when plasmid template representing 5 x 106 gene copies was tested, but it was evident (Ct values ≤ 30) in few instances when plasmid template representing 5 x 109 gene copies was utilized, the latter corresponding to the total gene copy number (as determined in this study) in 20 insects. The sensitivities of two assays (C. imicola, C. grisescens) were tested by spiking single insects into pools of 99 or 999, randomly selected non-target Ceratopogonidae (with approx. 90% Culicoides specimens). In the pools of 100, Ct values were in the range of those obtained with single insects when employing 1% of the isolated DNA, whereas the sensitivity with the pools of 1000 was low, presumably due to the low DNA concentrations obtained with a protocol that seems inadequate for these larger pools. Thus, the assays as described are applicable for the specific identification of biting midges in small pools. Primers and probes of this study were devised to be suitable for multiplexed assays but these evaluations await to be performed.

Cover Letter

Dear Editor,

Thank you very much for coordinating the review process of our manuscript which we have revised considering the comments/criticisms addressed by the two reviewers as specified in detail in the attached ‘Revision Note’. We would like to thank the reviewers for the time and efforts they put into the improvement of this manuscript. We hope that it is now suitable for publication in Parasitology.

We hope that the manuscript is now suitable for publication in Veterinary Parasitology.

Yours sincerely
Alexander Mathis

Molecular characterisation of Swiss Ceratopogonidae (Diptera) and evaluation of real-time PCR assays for the identification of *Culicoides* biting midges

Claudia E. Wenk, Christian Kaufmann, Francis Schaffner, Alexander Mathis

Reply to reviewers' comments

Reviewer Comments:
Editor in Chief: The manuscript is of interest. It should be revised following reviwers' issues and comments. Reviewer #3 tells of a file submitted for editing comments that authors should require to the Editorial Office. (attached)

Reviewer #2: Wenk et al. describe the development of a DNA-based assay to discriminate species of biting and non-biting midges, an assay that is desirable given the difficulties in morphological identification of these insects. The authors further utilized results from phylogenetic analysis of the mtCOI region to identify 2 cryptic Culicoides species and a potentially novel Culicoides sp. Overall, the manuscript is written well and the results contribute to a growing body of work aimed at molecular tools for species identification. I have the following questions/comments regarding the manuscript:

1-The numerous haplotypes per species that were identified by sequencing of the COI region plays a role in properly designing the primers/probes used for the described DNA-based assay. In some cases, the authors report that there were 10 - 11 haplotypes per species, yet specificity/cross-reactivity was only tested for 1 to 4 haplotypes. How do you account for potentially missing cross-reactivity as a result of the other haplotypes not being tested? Do polymorphisms that account for those other haplotypes possibly fall outside of the region that you're amplifying for the DNA-based assay? On that same line of thinking, it would be desirable to have a sequence alignment presented that identifies the locations of the designed primers/probe for at least one of the species (preferably one with numerous haplotypes).
REPLY: Primers and probes for a given species were designed at loci that were conserved among all haplotypes but differed from those of other species. However, degenerate primers/probes needed to be devised for some species, and this is shown in Table 2. Thus, we cannot exclude that additional haplotypes exist but Figure 1 illustrates that the variability of the species is limited, as sequences of specimens from England of Spain clustered well within the haplotypes determined in our study. Actually, our work to the best of our knowledge is the most comprehensive one with regard to considering haplotypes of species for the design of specific primers. As an example, in a very recently published duplex real time PCR for the simultaneous detection of C. obsoletus *and* C. scoticus *(Mathieu et al., Vet. Parasitol. 2011, article in press, this reference is also included in our manuscript) the design of the primers targeting noncoding (rRNA internal transcribed spacers) sequences was based on the corresponding sequences obtained from single specimens each (GenBank accession nrs. JF280792 and JF280793). Thus, albeit far from being perfect, our assays are well evaluated and indeed have the potential to be applied in further studies aiming at identifying biting midges.*
The reviewer wants us to include a sequence alignment, illustrating the locations of primers and probes. However, such an illustration would be arbitrary, and as the all gene sequences are available in GenBank (and those of the primers/probes in Table 2), such an alignment with the species specifically of interest can easily be done by a reader.

2-What were the correlation coefficients and slopes for your standard curves (to assess efficiency of your primers)? Was a dissociation protocol included to verify that a single product (single peak) was being amplified?

REPLY: We have tested serial dilutions (10e3, 10e4, 10e6) of some of the cloned targets, revealing PCR efficiencies around 94 % (e.g. C. scoticus*: correlation coefficient 0.994; slope -3.473, PCR efficiency 94.1%). All cloned targets were tested with 10e6 and 10e9 molecules (in the frame of the specificity testing), revealing Ct values of around 18 and 9.5, respectively, with higher Ct values (20.0, 10.9) for the assays using degenerate primers or probes (*C. chiopterus*,* C. griscescens*, as specified in Table 2). As the sensitivity seems not to be critical for detecting a target biting midge species among 99 others (as shown in our work) we feel that adding these data is of comparatively low relevance.*

No attempts were made to initially evaluate the primers' specificities with melt analyses (in additional separate assays using intercalating fluorescent dyes such as SYBR green), and this approach is not widely being applied when evaluating quantitative assays with Taqman probes.

3-A representative Ct graph is desirable for the data presented in lines 268-277. It would also be of interest to include un-spiked DNA pools to emphasize the ability of the assay to specifically detect the intended species.

REPLY: The key results of the spiking experiments are given in the text; adding one of the Ct graphs as a (colour) figure does not add to the quality of the data; rather, it is reminiscent of the Figures of ethidium-stained agarose gels of conventional PCRs appearing in publications some time ago (but such illustrations are no longer included).

No non-spiked pools were tested as it is very laborious to prepare such samples that are guaranteed free of the species to be tested (with the exception of C. imicola*, which does not occur in Switzerland; however, the PCR-detection of this species was not the primary focus).*

4-Inclusion of statements like those presented in lines 282 - 287 and lines 337-346 detract from the exciting discoveries you've made as a result of this research, especially what's discussed in section 4.2 and 4.3. The focus should be on the assay that you designed and on the phylogenetic analyses that provided some unique insight into a potentially novel species and the identification of 2 cryptic species.

REPLY: In lines 282-287, we mention the initial aim of the study (to develop a multiplex real-time PCR which allows to distinguish and quantify biting midges belonging to the three groups of Culicoides (Obsoletus, Pulicaris, others). Thorough analyses of the many mt COI sequences that were available to us (either from GenBank or determined during the study) revealed that this was not possible for us. Albeit a 'negative' result, we feel that this statement is justified as it is exactly the differentiation into these three groups that is of high practical relevance but obviously difficult to achieve with a PCR approach based on this barcoding mitochondrial gene sequence. Thus, this statement might advise caution to colleagues with this respectable aim in their minds.

Lines 337-346 mention that PCRs were devised to be used in multiplex assays (by choosing different fluorophores), but that due to a lack of time, we unfortunately were not able to thoroughly evaluate them (and preliminary data are not shown), which could be done in the future by ourselves or by colleagues interested in the application of a particular such multiplex PCR. Thus, this paragraph deserves to be kept in the manuscript (in the sense of an outlook).

5-Repeating the DNA isolation from the spiked pool of 1000 midges is desirable, especially since the results are due to an 'inadequate protocol'. If it can't be repeated to obtain optimum DNA concentrations for analysis, then it is preferred that presentation and discussion of those results be deleted. Again, it is distracting and detracts from the significance of what you're reporting. The results would still be relevant if reported as identification of biting midges in small pools.
REPLY: DNA isolations with pools of 1000 midges were done using a widely used standard protocol (which was efficient for DNA isolations from the smaller pools of 100 insects). The reasons for this discrepancy in efficiency are unclear (insufficient mechanical homogenization in the larger pools, saturation of the DNA binding columns, other). As we used a standard protocol, we feel that it is worthwhile to briefly communicate to the scientific community that a scaling-up of this protocol is not a trivial matter.

Some minor comments:
The language of the revised manuscript has been edited/amended according to the reviewer's suggestions as outlined below (no comment indicates that the reviewer's suggestion was adopted).

Throughout, substitute 'microscopic' for microscopical

Line 19 - 23: "The mt COI sequence was obtained from 380 Culicoides midges representing three regions in Switzerland, namely the Alps, Midland north of the Alps (Atlantic climate), and south of the Alps (Mediterranean climate). The same region was also sequenced from non-biting midges of the genera."
REPLY: We have modified this passage as follows (and feel that including a statement in the abstract on the purpose of the study is justified as the primers/probes investigated are ready to be used in such applications, albeit an evaluation in multiplexed format was not possible due to a lack of time). Further, to indicate the names of the non-biting midges investigated also is justified as we are not aware of another similar publication taking into account these related organisms, which commonly are by-catches in UV-light traps and which, as described in the manuscript, yield positive PCR results with primers allegedly specific for Culicoides*):*
'With the aim to develop real-time PCRs, partial mitochondrial cytochrome oxidase I gene (mt COI) sequences were obtained from 380 Culicoides *midges representing three regions of Switzerland, namely the Alps, Midland north of the Alps (Atlantic climate), and South of the Alps (Mediterranean climate). The same region was also sequenced from non-biting midges of the genera* Atrichopogon*,* Brachypogon*,* Dasyhelea*,* Forcipomyia *and* Serromyia*.'*

Line 25: "For each of.."
Lines 29 - 34: Sequence alignments of available mtCOI sequences from Ceratopogonids (Genbank, this study) were used to design real-time PCR primers and probes to distinguish C. chiopterus, C. deltus, .. Specificity of the primers was tested with cloned targets representing 1 to 4 haplotypes of 18 Culicoides spa. and 1 haplotype each from 4 other Ceratopogonids. No cross-reactivity was observed when plasmid template representing 5 x 10^6 gene copies was tested, but it was evident when plasmid template representing 5x10^9 gene copies was utilized, the latter corresponding to the total gene copy number in 20 insects.
Line 39: delete 'respectively'
Line 53: which --> that
Line 55: delete comma

Line 56: delete "by their"; "abundances, and experimental"

Line 71: Don't understand 'clinics' here.
REPLY: 'clinics' is replaced by 'clinical symptoms'.

Line 81: comprehensive knowledge of midge fauna
Line 83: The wing pattern allows classification of the insects into.
Line 84: delete 'groups' after Pulicaris since you already state 'vector-relevant groups'
Line 86: ".while others require microscopic analysis of slide-mounted insects".
Line 87: Morphological (delete The)
Line 97: SYBR

Line 102: delete "a huge number of"; and Line 102-103: "locus, and the locus demonstrates low."
REPLY: Lines 102, 102-103: this sentence is shortened and reformulated as follows:
'In contrast, sequence information available from the mt COI locus demonstrates low intra-specific divergence (haplotypes) and high inter-specific differences (Augot et al., 2010; Dallas et al., 2003; Linton et al., 2002; Pages et al., 2009).'

Line 113-114: Delete remainder of sentence after Switzerland
Line 136: and the thorax were stored in 2ml round-bottom Eppendorf tubes at -20C for DNA isolation.
Line 136: City, State information for Eppendorf

Line 162: "Amplicons were cloned using the Topo-TA." I believe the kit name has some registered trademark symbols? Please check for all kits described.
REPLY: We are not aware that Registered or TradeMark signs need to be included in Veterinary Parasitology papers (see e.g. Diaz-Lee et al., Vet. Parasitol. 176 (2011): 139-144).

Line 187-188: "The primers were PAGE purified and the MGB probes were HPLC purified" ?
REPLY: The sentence was clarified as follows: 'The ordinary Taqman probes were PAGE purified and the MGB probes were HPLC purified.'

Line 213: "midges and C. imicola specimens from Corsica (France) were treated similarly"

Line 228: "(most times)"?
REPLY: Clarified as follows: 'As the careful morphological analyses of these insects yielded different species identifications, including C. lupicaris *(most specimens),* C. lupicaris/pulicaris *intermediate phenotype, atypical* C. pulicaris *and* C. deltus*, the midges from this cluster were not considered a cryptic species but were tentatively named* Culicoides *sp.'*

Line 240: "and of new Culicoides sp"
We feel that the definitive article is necessary as the sentence refers to the particular new Culicoides *species (designated* Culicoides *sp.).*

Line 242: delete 'and', replace the comma before e.g.
Line 247: The 'sequence' data?

Line 254: of-->for
Line 256: of--> from

Reviwer #3: The authors present the results of a real-time PCR technique for the identification of Culicoides species from three different regions of Switzerland characterized by different altitudes. An explanation of the potentiality for this technique to be also used for the quantification of Culicoides specimens is given.
While of current interest and high relevance to Culicoides identification technique and update on species variability at high altitude, giving a contribute to the remit of Veterinary Parasitology, there are few corrections that must be addressed before the manuscript is suitable for publication.

1. The main objective of this paper is the real-time PCR technique for the identification of Culicoides species. Following the 'introduction', its length and content is not giving the right idea of what is the priority of this paper. Most of the information given in the first part of the 'introduction' are not relevant for this paper please see comments in the manuscript for suggestions/corrections.
REPLY: See below, reply to query A5. Briefly it might be justified to give in the introduction a somewhat broader picture of the topic, focusing on topics relevant for veterinary medicine.

2. The authors claim the discovering of two new species according to the rt-PCR developed in this work. Could the authors explain more about this in the manuscript under the 'discussion' section? It would be useful to have more details supporting this new discovering, such as collection of more specimens from that area such in order to find out the possibility of mixed population presence in that area.
REPLY: The discoveries of this new species (cryptic species of C. obsoletus *and* C. grisescens *as well as one, named* Culicoides *sp., with no close genetic relation to already described species) was a by-product of our genetic characterisation of the Swiss Culicoides fauna. The topic addressed by the reviewer, namely to collect more specimens and to characterise the composition of midges populations, e.g. in the Alpine environment, can be addressed by applying the assays described in our manuscript in future studies.*

3. The material and method section can be shortened and more suitable for a journal paper. Please see comments.
REPLY: We have adopted the suggested changes as specified under point 5 (see below).

4. Within the 'material and method', 'results' and 'discussion', there are repetitions that need to be cut. Especially in the 'results' part, there are paragraphs that must be moved in the discussion section for the way they are written and conclusion made as well.
REPLY: See replies to queries A9-A11, A13-A15 below (point 5).

5. Please check the english and make sure correction/suggestions are followed.
We have incorporated this reviewer's suggestions specified in a separate file ('Reviewer annotated MS Wenk et al Veterinary Parasitology11.doc', also included), if they clearly contribute to improve the content/grammar/language of the manuscript. Therefore, suggestions as the one shown below which is of purely stylistic nature were not considered (and many suggestions are grammatically incorrect or contain careless mistakes):

Our version (first sentence of abstract): 'Biting midges of the genus Culicoides (Diptera, Ceratopogonidae) are …'
Reviewer's suggestion: Biting midges belonging to Culicoides genus (Diptera, Ceratopogonidae) are …'

In particular, we replaced 'Ceratopogonids' by 'Ceratopogonidae'.

We specifically address the comments given in the annotated file as follows:

A1: It was not in Central Europe but in northern Europe. Please change it.
REPLY: OK (not being a geographer, it seems that 'north-western' Europe would be the most adequate designation).

A2: Same explained above
REPLY: Expression omitted by reformulation of the sentence (underlined): 'In northern Europe, biting midges (Diptera, Ceratopogonidae: Culicoides spp.) came to the fore as the vectors responsible for the unexpected and explosive spread of the bluetongue virus (BTV) that had been introduced <u>there</u> for the first time in 2006 (Carpenter et al., 2009)'.

A3: Please mentioned that it was discovered in goat as well, despite no clinical symptoms were displayed.
REPLY: Sentence amended as follows:
'… causing mild bluetongue-like symptoms in sheep and clinically unremarkable infections in goats …'

A4: The whole introduction is too long, moreover, the topic of this paper is on the use of molecular tools enable to identify Culicoides species and not about bluetongue disease. Please re-write it with giving maximum 8 rows on the disease and its relevance.
REPLY: The first paragraph does not focus on bluetongue disease as all as criticized; in fact, this disease is mentioned once in one sentence. In the other sentences of the paragraph, we provide an overview of other veterinary significances of biting midges, i.e. vectors of other, new or putatively emerging diseases, and also as causing dermatitis, which is of veterinary significance in many countries and actively being investigated. Thus, we give a broader overview on the veterinary significance of biting midges, and this seems to be justified for an article in 'Veterinary Parasitology', which has a readership with broad background and interests (and not only in entomology).

A5: Please quote reference (for morphological intraspecific variability):
REPLY: Reference inserted: '(see Pages et al., 2009)', in which this topic is summarized (including the literature).

A6: Please give more explanation on how this paper is going to give a bigger new contribute to what has been already published.
REPLY: The reviewer criticizes the last paragraph of the introduction ('The aim of this present study was to develop real-time PCRs for the identification of important biting midges of Switzerland, based on a thorough understanding of their genetic composition.') which seems adequate to describe the aims of the study.

A7: Please also quote other references (e.g. Delecolle, 1985, Kremer, 1965….)
REPLY: The reference Delecolle (1985), which is the basis for microscopical identification, is given at the correct position just a bit below; the older reference of Kremer (1965) not known to us and which was not used by us as an identification key needs not to be mentioned.

A8: Why do the author use this method? Is not clear from the text what is this chill table necessary for.
REPLY: The insects are homogenized in a mill and need to be cooled as they considerably heat up during the procedure. This should be known to persons familiar with mechanical homogenization.

A9: Is not necessary to specify these information. The text is long enough.
REPLY: It is necessary to give the suppliers of all the materials used, also of the primers and probes.

A10: Deletion for same reason mentioned above
REPLY: Chapter 2.6. occurs in duplicate in the file annotated by the reviewer (as do other parts of the manuscript in this file; this obviously happened while the reviewer was handling the file) which is not the case in the manuscript we submitted. Thus, the information criticized in comments A9 and A10 is not duplicated in our manuscript.

A11: Please move all these sentence to discussion.
REPLY: This sentence presents results and is adequately positioned.

A12: Please give an explanation of why these data are now shown.
REPLY: A second locus (rRNA gene ITS1) was analysed for specimens of C. obsoletus *and the* C. obsoletus *cryptic species to confirm their genetic distinctness. The key findings are given in the text, and a corresponding dendrogram is 'not shown' for reasons of space and as this is only a minor (confirmatory) result*.

A13: This should be moved to the discussion session
REPLY: Most of the contents of this paragraph are results which led to conclude that a new species, tentatively named Culicoides *sp., was discovered. Thus, we feel that this piece of information is correctly placed in the results section.*

A14: Please move this sentence to the discussion part.
REPLY: This sentence was deleted as this aspect indeed is already dealt with in the discussion section.

A15: This is a repetition of what already described in the material and method
REPLY: We agree and have shortened the sequences as suggested in the annotated file.

A16: Can the authors explain why the results are not shown?
REPLY: PCR-positive results were obtained with primers reported as being specific for the genus Culicoides *when using DNA of other genera of Ceratopogonidae; as it is not the focus of our work to evaluate the specificities of primers designed by others, the detailed results are not shown but two examples are specified in the text.*

A17: Degenerate?, A18: Please re-formulate this sentence, s not clear what the authors want to highlight.
REPLY: 'Degenerate' is indeed the correct expression ('degenerate primers'). However, the sentence was reformulated by replacing '… primers had to be designed degenerate … ' with '… degenerate primers had to be designed …'

A19: Maybe you meant above?
REPLY: No, we refer here to text given later in the manuscript.

A20: Please quote reference
REPLY: The conclusion given here is derived from our own data, thus no reference can be given.

A21: Please change this word, final is not clear
REPLY: 'Final criterion' replaced by 'decisive criterion'.

A22: Please re-formulate this sentence, is not clear what the authors want to say.
REPLY: Old version: 'Intra-specific morphological variation has been observed in various species (literature compiled in Pages et al., 2009), and obviously the Culicoides *sp. midges display variability in wing patterns causing uncertain identifications.'*
New version: 'Intra-specific morphological variation has been observed in various species (literature compiled in Pages et al., 2009), and obviously midges of the newly described species (designated Culicoides *sp.) display variability in wing patterns causing uncertain identifications.'*

A23-A25: Please quote reference
REPLY: The data are shown in Fig.1, and we refer to this figure in the text.

A26: Please give some references
REPLY: These data are shown in Fig. 1, and this is now also mentioned.

Molecular characterisation of Swiss Ceratopogonidae (Diptera) and evaluation of real-time PCR assays for the identification of *Culicoides* biting midges

Claudia E. Wenk, Christian Kaufmann, Francis Schaffner, Alexander Mathis*

Institute of Parasitology, University of Zurich, Winterthurerstr. 266a, 8057 Zurich, Switzerland.

* Corresponding author. Tel.: +41 44 635 85 01; fax: +41 44 635 89 07; *E-mail address*: alexander.mathis@uzh.ch (Alexander Mathis).

15 ABSTRACT

16 Biting midges of the genus *Culicoides* (Diptera, Ceratopogonidae) are vectors of several
17 viruses of veterinary relevance, and they can cause insect bite hypersensitivity. As the
18 morphological identification of these tiny insects is a difficult task in many cases, alternative
19 approaches are expedient. With the aim to develop real-time PCRs, we determined partial
20 mitochondrial cytochrome oxidase I gene (mt COI) sequences from 380 *Culicoides* midges
21 representing three regions of Switzerland, namely the Alps, Midland north of the Alps
22 (Atlantic climate), and South of the Alps (Mediterranean climate). The same region was also
23 sequenced from non-biting midges of the genera *Atrichopogon*, *Brachypogon*, *Dasyhelea*,
24 *Forcipomyia* and *Serromyia*. A total of 21 *Culicoides* species were identified by morphology.
25 Sequence variability (haplotypes) was observed in all species. For each of *C. grisescens* and
26 *C. obsoletus*, a novel cryptic species was identified. Whereas all individuals of *C. grisescens*
27 and of the cryptic *C. obsoletus* species (O2) originated only from Alpine sites, the known *C.*
28 *obsoletus* (O1) species was found in all three regions. Further, a sister taxon to *C. pulicaris*
29 was identified based on the mt COI sequences and named *Culicoides* sp. Alignments of
30 available mtCOI sequences from Ceratopogonidae (GenBank, this study) were used to
31 design real-time PCR primers and probes to distinguish *C. chiopterus*, *C. deltus*, *C. dewulfi*,
32 *C. grisescens* (including the cryptic species), *C. imicola*, *C. lupicaris*, *C. obsoletus* O1, *C.*
33 *obsoletus* O2, *C. pulicaris*, *C. scoticus* and *Culicoides* sp. Specificities of primers and probes
34 was tested with cloned targets representing 1 to 4 haplotypes of 18 *Culicoides* spp. and 1
35 haplotype each from 4 other Ceratopogonidae. No cross-reactivity was observed when
36 plasmid template representing 5×10^6 gene copies was tested, but it was evident (Ct values
37 ≤ 30) in few instances when plasmid template representing 5×10^9 gene copies was utilized,
38 the latter corresponding to the total gene copy number (as determined in this study) in 20
39 insects. The sensitivities of two assays (*C. imicola*, *C. grisescens*) were tested by spiking
40 single insects into pools of 99 or 999, randomly selected non-target Ceratopogonidae (with
41 approx. 90% *Culicoides* specimens). In the pools of 100, Ct values were in the range of

those obtained with single insects when employing 1% of the isolated DNA, whereas the sensitivity with the pools of 1000 was low, presumably due to the low DNA concentrations obtained with a protocol that seems inadequate for these larger pools. Thus, the assays as described are applicable for the specific identification of biting midges in small pools. Primers and probes of this study were devised to be suitable for multiplexed assays but these evaluations await to be performed.

Keywords:

Culicoides, vector identification, barcode, real-time PCR, specificity, cryptic species.

1. Introduction

In northern Europe, biting midges (Diptera, Ceratopogonidae: *Culicoides* spp.) came to the fore as the putative vectors responsible for the unexpected and explosive spread of the bluetongue virus (BTV) that had been introduced there for the first time in 2006 (Carpenter et al., 2009). A number of Palearctic species of *Culicoides* have been incriminated as BTV vectors based on virus isolations or detections by real-time PCR from field-caught midges, by host preferences and vector competence studies (summarized in (Pages et al., 2009). Biting midges are the biological vectors of several other viruses which are of relevance for Europe at present: The African horse sickness virus has repeatedly been observed and eradicated in Southern Europe, and new incursions are considered feasible (Mellor and Hamblin, 2004; Zimmerli et al., 2010). Similarly, the epizootic hemorrhagic disease virus which has a wide distribution in the world but has never been observed in Europe has recently expanded to countries neighboring Europe (Anonymous, 2009; Paweska et al., 2005; Yadin et al., 2008). Another virus, most probably transmitted by biting midges, is the Toggenburg orbivirus which has recently been discovered in Switzerland causing mild bluetongue-like symptoms in sheep and clinically unremarkable infections in goats (Chaignat et al., 2009; Hofmann et al., 2008). In addition to this newly recognized role as vectors in Europe, biting midges are a well-known nuisance pest in many parts of the continent and they can cause insect bite hypersensitivity (named colloquially as 'sweet itch') particularly in Equids. Candidate allergens have been characterized from the saliva of model species which can be reared in the laboratory (*C. sonorensis*, *C. nubeculosus*) (Hellberg et al., 2009; Langner et al., 2009; Schaffartzik et al., 2010; Wilson et al., 2008) but the species that cause the clinical symptoms under field conditions are not known (Sloet van Oldruitenborgh-Oosterbaan et al., 2009; van der Rijt et al., 2008).

Little data in terms of the *Culicoides* fauna composition is available for many regions of Europe. In Switzerland, there are 35 established (indigenous) valid species based on published data (Merz et al., 2006). However, several new species have recently been

reported for the first time from Switzerland, and a number of specimens could not unequivocally be identified by morphology (Cagienard et al., 2006; Casati et al., 2009; Kaufmann et al., 2009). In comparison, 51 species have been listed for north-eastern France, a region which has thoroughly been studied (Delécolle, 1985); Delécolle, personal communication). In order to study the significance of the various *Culicoides* species with regard to their role as vectors or elicitors of allergy, a comprehensive knowledge of midge fauna as well as tools for their rapid and easy identification are required. Identification at present is primarily carried out using morphological features. The observation of wing patterns allows the classification of the insects into vector-relevant groups such as Obsoletus and Pulicaris and other *Culicoides* spp. (Goffredo and Meiswinkel, 2004). Further, certain species can be identified based on wing pattern while others require microscopic analysis of slide-mounted parts of the bodies (Campbell and Pelham-Clinton, 1960; Delécolle, 1985). Morphological identification can therefore be a time-consuming procedure and is known to be a very difficult task in many cases even for expert taxonomists (Meiswinkel et al., 2008) due to faint characteristics or intraspecific variability (see Pages et al., 2009). Furthermore, the existence of cryptic species, i.e. morphologically similar midges that are genetically distinguishable, has recently been described (Pages et al., 2009).

Several PCR-based tests have been developed for the identification of *Culicoides* spp. targeting the ribosomal RNA genes internal transcribed spacer 1 or 2 (ITS-1, ITS-2) or the mitochondrial cytochrome oxidase I gene (mt COI, barcode region; (Waugh, 2007). The initial focus of these approaches was to detect *C. imicola* in pools of insects using a conventional PCR targeting ITS-1 (Cêtre-Sossah et al., 2004) which was further developed to real-time quantitative PCRs using SYBR green detection (Cêtre-Sossah et al., 2008; Monaco et al., 2010). Conventional and multiplexed ITS-1or ITS-2 PCRs were further developed for identifying individual insects from the Obsoletus group (Gomulski et al., 2005; Mathieu et al., 2011; Mathieu et al., 2007; Stephan et al., 2009). Relatively few data are available from these genetic loci, and considerable ITS-1 variation within one species has been

105 demonstrated (Ritchie et al., 2004). In contrast, sequence information available from the mt
106 COI locus demonstrates low intra-specific divergence (haplotypes) and high inter-specific
107 differences (Augot et al., 2010; Dallas et al., 2003; Linton et al., 2002; Pages et al., 2009).
108 Several conventional and multiplexed PCRs targeting this mt COI locus have been published
109 focusing on the detection of a number of *Culicoides* spp. from the Obsoletus and the
110 Pulicaris groups (Balczun et al., 2009; Nolan et al., 2007; Pages et al., 2009;
111 Schwenkenbecher et al., 2009).
112 Finally, another molecular technique (matrix-assisted laser desorption/ionization time of flight
113 mass spectrometry; MALDI-TOF MS) has very recently proven its potential for rapid, simple
114 and cost-effective characterization and identification of biting midges (Kaufmann et al.,
115 2011).
116 The aim of this present study was to develop real-time PCRs for the identification of
117 important biting midges of Switzerland.
118
119 **2. Materials and methods**
120 *2.1. Insects*
121 Insects were trapped in Switzerland according to the criteria described (Goffredo and
122 Meiswinkel, 2004) with Onderstepoort UV-light suction traps in the framework of a national
123 entomological monitoring programme (Kaufmann et al., 2009). For the present study, the
124 origin of the insects was classified as Alps (altitude 1000 – 2200 m; 7 trapping sites), Midland
125 (north of the Alps, altitude 400 – 700 m, Atlantic climate; 13 trapping sites) and South (south
126 of the Alps, altitude < 400 m; climate influenced by the Mediterranean Sea; 1 trapping site).
127 Specimens were randomly selected from different catches, and the number of individuals
128 investigated per trapping area roughly corresponds to the trapping effort. *Culicoides imicola*
129 specimens originated from Corsica (France). Prior to morphological identification and DNA
130 extraction, the midges were kept in 70% EtOH at 4 °C.
131

132 *2.2. Morphological identification*

133 Using a stereo microscope, the *Culicoides* spp. were separated from the other insects and
134 sorted into Obsoletus group, Pulicaris group and other *Culicoides* spp. based on wing
135 morphology (Goffredo and Meiswinkel, 2004). Species identification was achieved by
136 microscopic analyses of wing patterns and by the observation of body parts (head, legs,
137 wings and spermatheca of females or complete genitalia of males) of most specimens
138 mounted on slides according to (Delécolle, 1985). The remains of the abdomen and the
139 thorax were stored in 2 ml round-bottom Eppendorf tubes (Schönenbuch, Switzerland) at -20
140 °C for DNA isolation. Non-biting midges were identified to genus level according to (Wirth et
141 al., 1974)).

142

143 *2.3. DNA isolation*

144 Body parts (see above) or pools of midges were ground in 180 µl Tris-EDTA buffer (pH 8.4)
145 using a mixer mill (Retsch®, MM 300) with one (for single insects) or two (for pools of
146 insects) steel bead(s) (3 mm diameter) at 30 Hz for 1 min twice (three times for pooled
147 midges) with an in-between chill down step on ice. The homogenate was then incubated in a
148 heating block for 5 min at 95°C, and total DNA was isolated using the Qiamp DNA mini kit
149 (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. DNA
150 was eluted in 55 µl and was stored at -20 °C until further use.

151

152 *2.4. Conventional PCRs, cloning, sequencing and data analyses*

153 Part (585 bp) of the mitochondrial cytochrome oxidase subunit I gene (mt COI) was amplified
154 with the primers C1-J-1718_mod (5'-GGWGGRTTTGGWAAYTGAYTAG-3'), modified from a
155 primer described earlier and incorporating degenerate positions (Dallas et al., 2003), and
156 with the new primer CW1_R (5'-AGHWCCAAAAGTTTCYTTTTTCC-3') designed to be
157 insect-specific. The reaction volume of 50 µl consisted of 25 µl of the master mix (6 mM
158 $MgCl_2$) from the multiplex PCR kit (Qiagen), each primer at a concentration of 1 µM and 10 µl

159 template DNA. Amplifications were done in an automatic thermal cycler (DNA engine, MJ
160 Research, Bio-Rad Laboratories, Basel, Switzerland) with a profile including a HotStarTaq
161 DNA polymerase activation step (95 °C for 15 min) and 40 cycles at 95 °C for 30 s, annealing
162 at 50°C for 30 s and extension at 72 °C for 60 s. A final elongation step at 72 °C for 10 min
163 was included. PCRs with allegedly *Culicoides* genus-specific primers (PanCul F / PanCul R
164 targeting the rDNA ITS1 region; genF7 and COIR targeting the mt COI) were done as
165 described (Cêtre-Sossah et al., 2004; Schwenkenbecher et al., 2009).
166 Cloning of amplicons was achieved using the Topo TA cloning-vector pCR 2.1 (Invitrogen,
167 Carlsbad, CA) according to the manufacturer's manual. The plasmids were purified using the
168 Qiaprep spin miniprep kit (Qiagen) following the manufacturer's instructions. DNA
169 concentration was measured using a Nanodrop photometer (NanoDrop products,
170 Wilmington, USA), the plasmids diluted to stock solutions of 10^9 copies/µl and stored at -20
171 °C. Glycerol stocks of transfected bacteria in glycerol (Sigma, Buchs, Switzerland) are kept
172 at -80 °C.
173 Sequencing of amplicons, either directly after purification with the minelute PCR purification
174 kit (Qiagen) or after cloning, was done by a private company (Synergene GmbH, Schlieren,
175 Switzerland).
176 Dendrograms were inferred from sequences aligned with ClustalW and from Neighbor-
177 Joining (NJ) analyses using the software MEGA, version 4.1 (Tamura et al., 2007), with
178 default settings.
179
180 *2.5. Primer and probe design for species-specific real-time PCR assays*
181 Partial mt COI sequences determined in this study and corresponding ones retrieved from
182 GenBank were aligned using Multalin (Corpet, 1988). If necessary, the sequences were
183 manually adjusted. In a stepwise approach, consensus sequences of the species were
184 identified, and appropriate primers and probes were designed using the software Primer
185 Express 1.5TM. If required, the primers were designed degenerated to account for haplotype

variability and/or manually designed, and their annealing temperature was calculated using the program PerlPrimer. The primers and probes were tested for the formation of dimers and hairpins using AutoDimerv1 software (default settings). Oligonucleotides that had ΔG values higher than -5 kcal/mol at 37 °C were redesigned. Primers and probes were ordered at Microsynth AG (Balgach, Switzerland), except for the minor groove binding (MGB) probes which were synthesized by Applied Biosystems (Rotkreuz, Switzerland). The ordinary Taqman probes were PAGE purified and the MGB probes were HPLC purified.

2.6. Real-time PCRs, diagnostic parameters

PCRs were done in duplicates in 25 µl volumes including 12.5 µl iQ multiplex powermix (Bio-Rad Laboratories, Basel, Switzerland), 2.5 µl of each primer and probe and 5 µl template DNA. The reactions were run in an iCycler (Bio-Rad) in 96 well plates using the following cycling conditions: 95 °C for 3 min (hotstart), 35 cycles at 95 °C for 15 s and 60 °C for 1 min. All results were expressed in Ct values at a fixed threshold (default settings). The optimal concentrations of primers (50 nM, 300 nM or 900 nM) and probes (50 nM, 100 nM or 200 nM) were determined. The specificities of the primers and probes were tested with 5×10^6 and 5×10^9 copies of the cloned target of other *Culicoides* spp. and other Ceratopogonidae as template DNA (see table 2). The number of mt COI copies per individual insect was calculated for 4 *Culicoides* species (*C. scoticus*, *C. dewulfi*, *C. imicola* and *C. pulicaris*) based on standard curves obtained with three concentrations (5×10^6, 5×10^4 and 5×10^3 copies) of the corresponding cloned target sequence. The sensitivity of two assays was evaluated by analyzing five pools each of single target individuals (*C. imicola* or *C. grisescens*) added to 99 or 999 randomly selected other *Culicoides* spp. and approx. 10% non-biting midges before DNA extraction.

3. Results

3.1. Morphological and genetic identification of midges

A total of 380 *Culicoides* midges from three regions of Switzerland (Alps, Midland north of the Alps with Atlantic climate, South of the Alps with Mediterranean climate) were identified by microscopic analyses of mounted specimens and by determining partial sequences (465 – 541 bp) of the mt COI gene. In addition, 21 insects belonging to 5 genera of non-biting midges and *C. imicola* specimens from Corsica (France) were treated alike (Table 1). The biting midges (*Culicoides* spp.) could be assigned to 21 established species with a preponderance of members of the Obsoletus group, in particular *C. scoticus* (Table 1). For each *C. grisescens* and *C. obsoletus*, a novel cryptic species was identified, i.e. morphologically indistinguishable midges with distinctly different mt COI sequences (Figs. 1A and B; designation according to the suggested nomenclature for cryptic species of (Pages et al., 2009), as *C. grisescens* G1 and G2; *C. obsoletus* O1 and O2, respectively, for the established and the new cryptic species). Translational analyses of the sequences from *C. obsoletus* O1 and O2 using the invertebrate mitochondrial code revealed functional genes differing by 1-2 amino acids (aa) among the total 176 aa. Sequence analyses of a second locus (rDNA ITS1) showed low intra-species variability of the cloned amplicons from two individuals each of *C. obsoletus* O1 and O2 and a 2.8% inter-species diversity, placing the two species in different clusters in a dendrogram (not shown).

In addition, a genetically separate cluster of sequences was identified as a sister taxon to *C. pulicaris* (Fig. 1B). As the careful morphological analyses of these insects yielded different species identifications, including *C. lupicaris* (most specimens), *C. lupicaris*/*pulicaris* intermediate phenotype, atypical *C. pulicaris* and *C. deltus*, the midges from this cluster were not considered a cryptic species but were tentatively named *Culicoides* sp. The 12 haplotypes of the 34 individuals investigated divide into two branches (Fig 1B), but these do not reflect the morphological heterogeneity observed, as e.g. the 4 midges identified as *C. deltus* are found on both branches (nos. 360-363).

Around half of the species were collected from only one of the three regions, e.g. *C. deltus*, *C. grisescens* (both G1 and G2) and *C. obsoletus* O2 were only found in the Alps, whereas

240 *C. dewulfi* and *C. lupicaris* were identified from Midland traps only. Other species (*C.*
241 *chiopterus*, *C. obsoletus* O1, *C. pulicaris* and *C. scoticus*) were present in all three climate
242 regions. The genetic variability of important species (Obsoletus and Pulicaris groups, *C.*
243 *dewulfi*) and of the new *Culicoides* sp. (Table 1A) was further analyzed. A certain degree of
244 intra-species sequence variability (haplotypes) was observed in all species (Figs. 1A and B),
245 e.g. 10 haplotypes were determined for the 20 specimens *of C. lupicaris* and 11 haplotypes
246 for the 104 *C. scoticus* insects (for number of insects investigated see table 1). Within the
247 species that occurred in all three investigated areas (*C. chiopterus*, *C. obsoletus* O1, *C.*
248 *pulicaris* and *C. scoticus*), no obvious clustering of haplotypes according to the geographic
249 origin was observed. The sequence data are available in GenBank under accession numbers
250 HQ824371to HQ824525.
251
252 *3.2. Development of real-time PCRs*
253 Eleven real-time PCR assays targeting the mt COI gene were developed for the specific
254 identification of *Culicoides* (Tables 1A, 2): *C. chiopterus*, *C. deltus*, *C. dewulfi*, *C. grisescens*
255 (for both G1 and G2), *C. imicola*, *C. lupicaris*, *C. obsoletus* O1, *C. obsoletus* O2, *C. pulicaris*,
256 *C. scoticus*, *Culicoides* sp. Primers and probes were designed in silico by considering all
257 sequence information available (GenBank, this study) for Ceratopogonidae. The optimal
258 concentrations for primers and probes were assessed (table 2), and the specificities of the
259 assays were evaluated by PCRs with cloned targets (n=40) of 1 to 4 haplotypes from 18
260 *Culicoides* spp. and of 1 haplotype each of 4 other Ceratopogonidae which are regularly
261 found in the UV-light traps (Table 2). Hence, when testing 5×10^6 targets of the appropriate
262 species, Ct values between 18.6 and 20 were observed, and all tests with targets from other
263 species were negative. Using 1000-fold higher concentrations of target molecules (5×10^9)
264 yielded Ct values of 8.5 - 10.5 for the proper targets, and cross-reactivity (Ct values ≤ 30)
265 was observed in a few instances (Table 2).

266 The number of targets (mitochondrions) in single insects was evaluated for *C. dewulfi*, *C.*
267 *imicola*, *C. pulicaris* and *C. scoticus*, based on standard curves obtained with the respective
268 cloned sequences. Hence, 3.4 x 10^7 to 2.8 x 10^8 copies per single biting midge were
269 calculated.
270 Finally, the sensitivities of the two assays for detection of *C. imicola* and *C. grisescens* were
271 tested with spiked pools. DNA concentrations obtained from these pools ranged from 107 to
272 130 ng/µl (100 insects/pool), but were as low as 14 to 39 ng/µl for the larger pools (1000
273 insects). Real-time PCRs with 5 µl DNA solutions from the pools of 100 mostly were
274 negative. These tests were repeated with 0.5 µl DNA solutions (i.e. 5 µl of a 1:10 dilution).
275 Hence, one *C. imicola* or *C. grisescens* specimen in total 100 midges was detectable with Ct
276 values between 20.8 - 22.6 (*C. imicola*) or 21.9 - 23.9 (*C. grisescens*); these values being
277 between 22.9 - 28.4 or 26.1 - 30.9, respectively, when investigating undiluted DNA from the
278 pools containing 1000 insects.
279
280 **4. Discussion**
281 *4.1. Real-time PCRs*
282 *Culicoides* specimens usually are pre-sorted by morphological features into Obsoletus group,
283 Pulicaris group and other *Culicoides* spp. The very initial aim of the present study was to
284 develop a triplex real-time PCR to accomplish the same task, with the intrinsic added
285 potential value of also gaining quantitative estimates of the composition of insect pools.
286 However, the extensive analyses of huge numbers of mt COI sequences from *Culicoides*
287 spp., either retrieved from GenBank or determined during this study, revealed that this posed
288 an unsolvable challenge for us. Hence, we focused on developing real-time PCR assays for
289 the identification of specific *Culicoides* biting midges (Tables 1A, 2). For the first time, also
290 corresponding sequences of non-biting midges, which are usual by-catches in light traps,
291 were determined and taken into consideration when designing the *Culicoides* primers and
292 probes. The necessity of this approach is illustrated by the fact that primers that were

293 described as being specific for the genus *Culicoides* also amplify DNA from the non-biting
294 midges investigated in this study (see Table 1; results not shown). Hence, the primers
295 genF7/COIR (Schwenkenbecher et al., 2009) targeting the mt COI have identical or highly
296 similar sequences to the respective gene sequences of these non-biting midges, and the
297 expected amplicon of 104 bp was obtained when performing that particular PCR assay e.g.
298 with DNA from *Forcipomyia* sp. Further, supposedly genus-specific primers targeting the 18S
299 and 5.8S rRNA genes and flanking the ITS1 (Cêtre-Sossah et al., 2004) readily produced
300 amplicons in the range of 300 – 550 bp with DNA from the non-biting midges.
301 The existence of genetic variants (haplotypes) was found in all investigated species. For
302 some of the target midges, degenerate primers had to be designed to account for this
303 sequence heterogeneity at those gene sequence regions which were identified as suitable
304 markers for a species (by reasonably differing from all other available sequences). Further,
305 as the target gene is rather rich in A/T, minor groove binding probes were chosen in some
306 instances to allow for a high specificity of shorter oligonucleotides (Yao et al., 2006).
307 Specificities of the assays were tested with two concentrations of cloned targets of 22 other
308 midge species, with several haplotypes being tested for some species. Hence, no cross
309 reactivities of the assays were observed when investigating 5 x 10^6 targets, whereas the
310 1000 fold higher concentrations yielded weak reactions in some instances. These cross-
311 reactions, which interestingly mostly occurred only with a single haplotype of a species, were
312 rather unexpected, as primers and probes were designed with several mismatches to non-
313 target sequences, particularly at the 3' end and in the middle, respectively. For example the
314 probe of *C. scoticus* has 6 mismatches (length of probe: 27nt) and both primers 2
315 mismatches with the sequence of the cloned haplotype of *C. chiopterus* with which a Ct
316 value of 26 was obtained in the real-time PCR spiked with 5 x 10^9 targets (Table 2).
317 However, the number of non-target DNA used in these assays (5 x 10^9) was very high,
318 corresponding to the total number of target genes of at least 20 such non-target midges
319 (which contain between 3.4 x 10^7 to 2.8 x 10^8 copies of the gene). As outlined below, the total

DNA of this number of insects in a single assay might be too high and cause inhibition of the reaction. Hence, these cross reactivities might be of little concern for practical purposes, and it must be stressed that such evaluations of other published PCR assays were done with DNA aliquots obtained from single biting midges of mostly a limited number only of non-target *Culicoides* species.

Real-time PCRs with DNA from pools of 100 midges for the detection of a single spiked target species was successful when employing 1% of the DNA but were hampered when using 10%, probably due to PCR inhibition. By using 1% of the DNA, approx. 10^6 targets of the spiked individual are present in the reactions. The Ct values in these assays were around 21 for the detection of *C. imicola* which very well match the value of around 19 - 20 when testing 5 x 10^6 cloned targets (Table 2). Hence, this assay is highly sensitive and specific for the detection of a single midge in pools of at least 100. In contrast, DNA isolation from pools of 1000 midges yielded low concentrations, and the protocol applied needs to be improved. However, investigating larger pools of midges might in a few cases give false-positive results, e.g. when using the *C. chiopterus* assay with DNA obtained from pools containing huge numbers of *C. scoticus* (Table 2). Thus, our approach is applicable for the specific identification of midges in pools of less than 1000 midges.

The primers and probes of this study were devised to be suitable for multiplexed assays, i.e. all were analyzed for the formation of dimers with any other oligonucleotide and redesigned if necessary. The fluorophores of the probes (Table 2) were chosen to allow triplex assays e.g. a ‘bluetongue panel’ targeting the major vector species *C. imicola*, *C. obsoletus* O1 and *C. scoticus*, a ‘Pulicaris group panel’ covering *C. pulicaris*, *C. lupicaris*, and *C. grisescens* (both G1 and G2), or an ‘alpine panel’ including *C. deltus*, *C. obsoletus* O2 and *Culicoides* sp. which nearly exclusively have been collected at higher altitudes. However, these investigations have not yet been accomplished, and other combinations with the single assays described (Table 2) are feasible, possibly after adjusting the fluorophores.

347 *4.2. Cryptic species*/Culicoides *sp.*

348 The analyses of partial mt COI sequences confirmed earlier findings (Augot et al., 2010;
349 Calvo et al., 2009; Dallas et al., 2003; Linton et al., 2002; Pages et al., 2009; Pages and
350 Sarto, 2005) that this locus displays low intra-specific variation and considerable inter-
351 specific variation in *Culicoides* spp. These analyses also revealed the presence of two new
352 cryptic species, morphologically indistinguishable from *C. grisescens* and *C. obsoletus*,
353 respectively, but differing by 10-11% at nucleotide level of the mt COI locus. Such cryptic
354 species have recently been described for the first time in the genus *Culicoides* in three (*C.*
355 *fagineus*, *C. newsteadi* and *C. pulicaris*) of five investigated species (Pages et al., 2009) by
356 analyzing the same genetic locus as addressed in our study. An alternative explanation of
357 this observed genetic variability could be the existence of mitochondrial DNA sequences that
358 integrated in the nuclear genome (nuclear mtDNA, 'NUMT') and evolved as pseudogenes.
359 Such NUMT elements have been identified in some insects (Sunnucks and Hales, 1996;
360 Zhang and Hewitt, 1996), but recent analyses of insect genomes have revealed that in
361 general they are scarce. The two hitherto recognized exceptions among insects are the
362 honey bee *Apis mellifera* and the yellow fever mosquito *Aedes aegypti* which have plenty of
363 NUMTs (summarized in (Black and Bernhardt, 2009). Translational analyses of the mt COI
364 sequences of the cryptic species *C. grisescens* G2 and *C. obsoletus* O2 confirmed that these
365 novel sequences encode for proteins (i.e. no frame shifts or stop codons present). In
366 addition, analyses of *C. obsoletus* O2 at a second locus (rDNA ITS) confirmed its
367 distinctness from and relatedness to *C. obsoletus* O1. Further, real-time PCRs with primers
368 and probes targeting *C. obsoletus* O1 were negative on DNA from 2 individuals identified as
369 *C. obsoletus* O2, and vice versa (not shown). Finally, mass spectrometric analyses by
370 MALDI-TOF allow to differentiate between *C. grisescens* G1 and G2 (own unpublished data;
371 *C. obsoletus* not investigated).

372 A puzzling finding was the variability of the morphological species designation of specimens
373 which genetically clustered as sister taxon of *C. pulicaris* and which were named *Culicoides*

374 sp. For the morphological identification of midges species belonging to the Pulicaris group,
375 the decisive criterion using currently available identification keys (Delécolle, 1985) is the wing
376 pattern, all other features considered being non-discriminative. Intra-specific morphological
377 variation has been observed in various species (literature compiled in (Pages et al., 2009),
378 and obviously midges of the newly described species (designated *Culicoides* sp.) display
379 variability in wing patterns causing uncertain identifications. Closer morphometric analyses of
380 these items might allow devising a more precise key with reliable parameters. A similar
381 approach has recently been described for the differentiation of *C. obsoletus* and *C. scoticus*
382 females which were considered undistinguishable (Augot et al., 2010). A cryptic species of *C.*
383 *pulicaris*, designated *C. pulicaris* P3, has recently been identified (Pages et al., 2009). These
384 specimens were morphologically not discriminable from *C. pulicaris* and their mt COI
385 sequences considerably differ (not shown) from those of the *Culicoides* sp. identified in
386 Switzerland. Hence, based on the genetic data and on the morphological ambivalence,
387 *Culicoides* sp. indeed seems to be a novel species.
388
389 *4.3. Geographical distribution of species/haplotypes*
390 Several *Culicoides* species have only been found in one of the three investigated climatic
391 regions of Switzerland (Alps, midland north of the Alps with Atlantic climate, region south of
392 the Alps with Mediterranean climate), but, clearly, the number of investigated specimens is
393 too low to draw conclusions on their definitive geographic distribution. Interestingly, both
394 discovered cryptic species (*C. grisescens* G2, *C. obsoletus* O2) were only sampled in the
395 Alpine region; from the new species *Culicoides* sp. all but one specimen also originated from
396 higher altitudes. It remains to be elucidated whether these species are truly adapted to this
397 climate.
398 Specimens from 4 species (*C. chiopterus*, *C. obsoletus* O1, *C. pulicaris* and *C. scoticus*)
399 were collected in all three climatic regions, and no clustering of haplotypes according to the
400 geographic origin was obvious. Thus, the alpine crest seems not to be a barrier for the

dispersal of these midges. Further, co-clustering of mt COI sequences with those from Swiss midges is observed for sequences from Spain (*C. pulicaris*, *C. scoticus*), Great Britain (*C. pulicaris, C. grisescens* G1, *C. scoticus*, *C. chiopterus*, *C. dewulfi*) and from Bulgaria (*C. obsoletus* O1) (Figs. 1A and B), indicating a lack of barriers for these species in Europe. In contrast, Spanish *C. obsoletus* O1 and *C. lupicaris* are placed on separate branches, supported by high bootstrap values (Fig. 1). Therefore, the population genetic structure of the investigated midge species might differ across Europe, and this is of ecological and epidemiological significance, particularly with regard to vector competence.

Acknowledgements

We kindly thank Jeannine Hauri for her excellent technical support in the lab and all the farmers for carefully and reliably running the light traps. Furthermore, we are indebted to Dr. J.-C. Delécolle (Université Louis Pasteur, Strasbourg) for his inestimable assistance in the morphological identification of many of our trapped Ceratopogonidae biting midges and for providing the *C. imicola* specimens. This study was funded by the Swiss Federal Veterinary Office (project No. 1.08.10) and represents the doctoral thesis of Claudia E. Wenk, veterinarian.

References

References

Anonymous, 2009. Scientific opinion on epizootic hemorrhagic disease. EFSA Journal 7(12), Document 1418, 67 pp.

Augot, D., Sauvage, F., Jouet, D., Simphal, E., Veuille, M., Couloux, A., Kaltenbach, M.L., Depaquit, J., 2010. Discrimination of *Culicoides obsoletus* and *Culicoides scoticus*, potential bluetongue vectors, by morphometrical and mitochondrial cytochrome oxidase subunit I analysis. Infect. Genet. Evol. 10, 629-637.

427 Balczun, C., Vorsprach, B., Meiser, C.K., Schaub, G.A., 2009. Changes of the abundance of
428 *Culicoides obsoletus* s.s. and *Culicoides scoticus* in Southwest Germany identified by
429 a PCR-based differentiation. Parasitol. Res. 105, 345-349.
430 Black, W.C., Bernhardt, S.A., 2009. Abundant nuclear copies of mitochondrial origin
431 (NUMTs) in the *Aedes aegypti* genome. Insect Mol. Biol. 18, 705-713.
432 Cagienard, A., Griot, C., Mellor, P.S., Denison, E., Stärk, K.D.C., 2006. Bluetongue vector
433 species of *Culicoides* in Switzerland. Med. Vet. Entomol. 20, 239-247.
434 Calvo, J.H., Calvete, C., Martinez-Royo, A., Estrada, R., Miranda, M.A., Borras, D., Sarto,
435 I.M.V., Pages, N., Delgado, J.A., Collantes, F., Lucientes, J., 2009. Variations in the
436 mitochondrial cytochrome c oxidase subunit I gene indicate northward expanding
437 populations of *Culicoides imicola* in Spain. Bull. Entomol. Res. 99, 583-591.
438 Campbell, J.A., Pelham-Clinton, E.C., 1960. A taxonomic review of the British species of
439 *Culicoides* Latreille (Diptera, Ceratopogonidae). Proc. R. Soc. Edinburgh, Section B
440 67, 181-302.
441 Carpenter, S., Wilson, A., Mellor, P.S., 2009. *Culicoides* and the emergence of bluetongue
442 virus in northern Europe. Trends Microbiol. 17, 172-178.
443 Casati, S., Racloz, V., Delécolle, J.-C., Mathis, A., Griot, C., Stärk, K.D.C., Vanzetti, T., 2009.
444 An investigation on the *Culicoides* species composition at seven sites in southern
445 Switzerland. Med. Vet. Entomol 23, 93-98.
446 Cêtre-Sossah, C., Baldet, T., Delécolle, J.-C., Mathieu, B., Perrin, A., Grillet, C., Albina, E.,
447 2004. Molecular detection of *Culicoides spp.* and *Culicoides imicola*, the principal
448 vector of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe.
449 Vet. Res. 35, 325-337.
450 Cêtre-Sossah, C., Mathieu, B., Setier-Rio, M.L., Grillet, C., Baldet, T., Delécolle, J.-C.,
451 Albina, E., 2008. Development and evaluation of a real-time quantitative PCR assay
452 for *Culicoides imicola*, one of the main vectors of bluetongue (BT) and African horse
453 sickness (AHS) in Africa and Europe. Res. Vet. Sci. 85, 372-382.

454 Chaignat, V., Worwa, G., Scherrer, N., Hilbe, M., Ehrensperger, F., Batten, C., Cortyen, M.,
455 Hofmann, M., Thuer, B., 2009. Toggenburg orbivirus, a new bluetongue virus: initial
456 detection, first observations in field and experimental infection of goats and sheep.
457 Vet. Microbiol. 138, 11-19.
458 Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids
459 Res. 16, 10881-10890.
460 Dallas, J.F., Cruickshank, R.H., Linton, Y.M., Nolan, D.V., Patakakis, M., Braverman, Y.,
461 Capela, R., Capela, M., Pena, I., Meiswinkel, R., Ortega, M.D., Baylis, M., Mellor,
462 P.S., Mordue (Luntz), A.J., 2003. Phylogenetic status and matrilineal structure of the
463 biting midge, *Culicoides imicola*, in Portugal, Rhodes and Israel. Med. Vet. Entomol.
464 17, 379-387.
465 Delécolle, J.-C., 1985. Nouvelle contribution à l'étude systématique et iconographique des
466 espèces du genre *Culicoides* (Diptera: Ceratopogonidae) du Nord-Est de la France.
467 Université Louis Pasteur, Strasbourg, 238 pp., Reissue 1993.
468 Goffredo, M., Meiswinkel, R., 2004. Entomological surveillance of bluetongue in Italy:
469 methods of capture, catch analysis and identification of *Culicoides* biting midges. Vet.
470 Ital. 40, 260-265.
471 Gomulski, L.M., Meiswinkel, R., Delécolle, J.-C., Goffredo, M., Gasperi, G., 2005.
472 Phylogenetic relationships of the subgenus Avaritia Fox, 1955 including *Culicoides*
473 *obsoletus* (Diptera, Ceratopogonidae) in Italy based on internal transcribed spacer 2
474 ribosomal DNA sequences. Syst. Entomol. 30, 619-631.
475 Hellberg, W., Mellor, P.S., Torsteinsdottir, S., Marti, E., 2009. Insect bite hypersensitivity in
476 the horse: comparison of IgE-binding proteins in salivary gland extracts from
477 *Simulium vittatum* and *Culicoides nubeculosus*. Vet. Immunol. Immunopathol. 132,
478 62-67.

Hofmann, M.A., Renzullo, S., Mader, M., Chaignat, V., Worwa, G., Thuer, B., 2008. Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. Emerg. Infect. Dis. 14, 1855-1861.

Kaufmann, C., Schaffner, F., Mathis, A., 2009. Monitoring of biting midges (*Culicoides* spp.), the potential vectors of the bluetongue virus the 12 climatic regions of Switzerland. Schweiz. Arch. Tierheilkd. 5, 205-513.

Kaufmann, C., Ziegler, D., Schaffner, F., Carpenter, S., Pflüger, V., Mathis, A., 2010. Evaluation of matrix-assisted laser desorption/ionization time of flight mass spectrometry for characterization of *Culicoides nubeculosus* biting midges. Med. Vet. Entomol., 25, 32-38.

Langner, K.F., Jarvis, D.L., Nimtz, M., Heselhaus, J.E., McHolland, L.E., Leibold, W., Drolet, B.S., 2009. Identification, expression and characterisation of a major salivary allergen (Cul s 1) of the biting midge *Culicoides sonorensis* relevant for summer eczema in horses. Int. J. Parasitol. 39, 243-250.

Linton, Y.M., Mordue (Luntz), A.J., Cruickshank, R.H., Meiswinkel, R., Mellor, P.S., Dallas, J.F., 2002. Phylogenetic analysis of the mitochondrial cytochrome oxidase subunit I gene of five species of the *Culicoides imicola* species complex. Med. Vet. Entomol. 16, 139-146.

Mathieu, B., Delecolle, J.-C., Garros, C., Balenghien, T., Setier-Rio, M.-L., Candolfi, E., Cêtre-Sossah, C., 2011. Simultaneous quantification of the relative abundance of species complex members: Application to *Culicoides obsoletus* and *Culicoides scoticus* (Diptera: Ceratopogonidae), potential vectors of bluetongue virus. Vet. Parasitol., doi:10.1016/j.vetparas.2011.05.052 (corrected proof).

Mathieu, B., Perrin, A., Baldet, T., Delecolle, J.-C., Albina, E., Cêtre-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, 1019-1025.

506 Meiswinkel, R., Baldet, T., de Deken, R., Takken, W., Delécolle, J.-C., Mellor, P.S., 2008.
507 The 2006 outbreak of bluetongue in northern Europe - the entomological perspective.
508 Prevent. Vet. Med. 87, 55-63.
509 Mellor, P.S., Hamblin, C., 2004. African horse sickness. Vet. Res. 35, 445-466.
510 Merz, B., Bächli, G., Haenni, J.-P., 2006. Zweiter Nachtrag zur Ckeckliste der Diptera der
511 Schweiz. Mitt. Schweiz. Entomol. Gesell. 56, 135-165.
512 Monaco, F., Benedetto, L., Di Marcello, V., Lelli, R., Goffredo, M., 2010. Development and
513 preliminary evaluation of a real-time polymerase chain reaction for the identification of
514 *Culicoides obsoletus* sensu strictu, *C. scoticus* and *C. montanus* in the Obsoletus
515 complex in Italy. Vet. Ital. 46, 209-214.
516 Nolan, D.V., Carpenter, S., Barber, J., Mello, P.S., Dallas, J.F., Mordue, J.A., Piertney, S.B.,
517 2007. Rapid diagnostic PCR assay for members of the *Culicoides obsoletus* and
518 *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in
519 Europe. Vet. Microbiol. 124, 82-94.
520 Pages, N., Munoz-Munoz, F., Talavera, S., Sarto, V., Lorca, C., Nunez, J.I., 2009.
521 Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the
522 subgenus *Culicoides* and development of species-specific PCR assays based on
523 barcode regions. Vet. Parasitol. 165, 298-310.
524 Pages, N., Sarto, I.M.V., 2005. Differentiation of *Culicoides obsoletus* and *Culicoides*
525 *scoticus* (Diptera: Ceratopogonidae) based on mitochondrial cytochrome oxidase
526 subunit I. J. Med. Entomol. 42, 1026-1034.
527 Paweska, J.T., Venter, G.J., Hamblin, C., 2005. A comparison of the susceptibility of
528 *Culicoides imicola* and *C. bolitinos* to oral infection with eight serotypes of epizootic
529 haemorrhagic disease virus. Med. Vet. Entomol. 19, 200-207.
530 Ritchie, A., Blackwell, A., Malloch, G., Fenton, B., 2004. Heterogeneity of ITS1 sequences in
531 the biting midge *Culicoides impunctatus* (Goetghebuer) suggests a population in
532 Argyll, Scotland, may be genetically distinct. Genome 47, 546-558.

533 Schaffartzik, A., Marti, E., Torsteinsdottir, S., Mellor, P.S., Crameri, R., Rhyner, C., 2010.
534 Selective cloning, characterization, and production of the *Culicoides nubeculosus*
535 salivary gland allergen repertoire. Vet. Immunol. Immunopathol. (in press).
536 Schwenkenbecher, J.M., Mordue, A.J., Switek, K., Piertney, S.B., 2009. Discrimination of
537 *Culicoides* midge larvae using multiplex polymerase chain reaction assays based on
538 DNA sequence variation at the mitochondrial cytochrome C oxidase I gene. J. Med.
539 Entomol. 46, 610-614.
540 Sloet van Oldruitenborgh-Oosterbaan, M.M., van Poppel, M., de Raat, I.J., van den Boom,
541 R., Savelkoul, H.F., 2009. Intradermal testing of horses with and without insect bite
542 hypersensitivity in The Netherlands using an extract of native *Culicoides* species. Vet.
543 Dermatol. 20, 607-614.
544 Stephan, A., Clausen, P.H., Bauer, B., Steuber, S., 2009. PCR identification of *Culicoides*
545 *dewulfi* midges (Diptera: Ceratopogonidae), potential vectors of bluetongue in
546 Germany. Parasitol. Res. 105, 367-371.
547 Sunnucks, P., Hales, D.F., 1996. Numerous transposed sequences of mitochondrial
548 cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). Mol.
549 Biol. Evol. 13, 510-524.
550 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular evolutionary genetics
551 analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.
552 van der Rijt, R., van den Boom, R., Jongema, Y., van Oldruitenborgh-Oosterbaan, M.M.,
553 2008. *Culicoides* species attracted to horses with and without insect hypersensitivity.
554 Vet. J. 178, 91-97.
555 Waugh, J., 2007. DNA barcoding in animal species: progress, potential and pitfalls.
556 Bioessays 29, 188-197.
557 Wilson, A.D., Heesom, K.J., Mawby, W.J., Mellor, P.S., Russell, C.L., 2008. Identification of
558 abundant proteins and potential allergens in *Culicoides nubeculosus* salivary glands.
559 Vet. Immunol. Immunopathol. 122, 94-103.

560 Wirth, W.W., Ratanaworabhan, N.C., Blanton, F.S., 1974. Synopsis of the genera of
561 Ceratopogonidae (Diptera). Ann. Parasitol. Hum. Comp. 49, 595-613.
562 Yadin, H., Brenner, J., Bumbrov, V., Oved, Z., Stram, Y., Klement, E., Perl, S., Anthony, S.,
563 Maan, S., Batten, C., Mertens, P.P., 2008. Epizootic haemorrhagic disease virus type
564 7 infection in cattle in Israel. Vet. Rec. 162, 53-56.
565 Yao, Y., Nellaker, C., Karlsson, H., 2006. Evaluation of minor groove binding probe and
566 Taqman probe PCR assays: Influence of mismatches and template complexity on
567 quantification. Mol. Cell. Probes 20, 311-316.
568 Zhang, D.-X., Hewitt, G.M., 1996. Nuclear integrations: challenges for mitochondrial DNA
569 markers. Trends Ecol. Evol. 11, 247-251.
570 Zimmerli, U., Herholz, C., Schwermer, H., Hofmann, M., Griot, C., 2010. Afrikanische
571 Pferdepest und equine Encephalosis: Muss sich die Schweiz vorbereiten? Schweiz.
572 Arch. Tierheilkd. 152, 165-175.
573
574
575
576
577

578 Figure 1. Dendrograms inferred from partial mitochondrial COI gene sequences of *Culicoides*
579 spp. from A. the Obsoletus group (*C. obsoletus, C. scoticus*, *C. chiopterus*) and *C. dewulfi*
580 and B. the Pulicaris group (*C. pulicaris*, *C. lupicaris*, *C. deltus*, *C. grisescens*) and *Culicoides*
581 sp. Depicted are all haplotypes identified in the three investigated regions in Switzerland
582 (Alps, Midland north of the Alps, South of the Alps). The number of isolates analyzed per
583 taxonomic unit is given in Table 1. Included are single sequences from *C. imicola* (C. imi),
584 *Forcipomyia* sp. (Forc. sp.), *C. pulicaris* (C. pul) (Fig 1A), *C. obsoletus* (C. obs) (Fig 1B) and
585 corresponding *Culicoides* GenBank entries if available. Sequences were aligned with
586 ClustalW and the tree was deduced from Neighbour-Joining (NJ) analyses using the
587 software MEGA, version 4.1 (Tamura et al., 2007). Bootstrap values are given on the nodes.
588 Sequences from GenBank are supplemented with the country origin of the midge (BG
589 Bulgaria, ES Spain, GB Great Britain).

Table

Table 1: Trapping regions and number of midges (Ceratopogonidae) investigated. *Culicoides* identification was based on morphological and/or genetic (partial sequence of mt COI gene) analyses.

Species	Trapping regions in Switzerland[1]		
A. *Culicoides* for which real-time PCRs were evaluated in this study[2]	Alps	Midland	South
C. chiopterus	2	25	1
C. deltus	7	0	0
C. dewulfi	0	5	0
C. grisescens G1[3]	7	0	0
C. grisescens G2[3]	12	0	0
C. lupicaris	0	20	0
C. obsoletus O1	7	57	4
C. obsoletus O2	9	0	0
C. pulicaris	2	13	11
C. scoticus	5	97	2
Culicoides sp.	33	1	0
B. Other *Culicoides* spp.			
C. brunnicans	3	0	0
C. circumscriptus	0	4	4
C. comosioculatus	4	2	0
C. duddinstoni	0	1	0
C. fascipennis	5	1	0
C. festivipennis	0	2	6
C. furcillatus	0	3	0
C. jurensis	3	0	0
C. kibunensis	0	0	1
C. pallidicornis	0	3	7
C. punctatus	0	8	1
C. reconditus	1	0	0
C. segnis	1	0	0
C. Non-biting Ceratopogonidae			
Atrichopogon sp.	1	0	0
Brachypogon spp.	2	0	0
Dasyhelea spp.	0	2	0
Forcipomyia spp.	0	15	0
Serromyia sp.	0	1	0

[1]Alps (altitude 1000 – 2200 m), Midland (north of the Alps, altitude 400 – 700 m, Atlantic climate) and South (south of the Alps, altitude < 400 m; climate influenced by the Mediterranean Sea).

[2]In addition, a real-time PCR was designed and evaluated for *C. imicola* of which individuals originating from Corsica (France) were available (kindly provided by J.-C. Delécolle).

[3]PCR assay designed to be specific for both *C. grisescens* G1 and G2.

Table

1 Table 2: Sequences of primers and probes (5' - 3') for real-time PCR assays, and cross-reactivities observed.

Attempted specificity	Primers and probes (optimal concentration in nM)		Cross-reactivity[1)] with other *Ceratopogonidae*[2)] (Ct value)
C. chiopterus	chi_F chi_R chi_P	AGGTATTAGTTCTATTTTAGGGGCT (300) AATGATAAAAGRAGTAAAATTGCAGTKAGA (300) FAM -CTATTATTAATATACGTTCTAATGGAATAAC-NFQ-MGB (100)	*C. imicola* (28), *C. scoticus* (30)
C. deltus	del_F del_R del_P	TGGAACTGGATGAACCGTA (300) AGAAGAAATYCCTGCTAAATGTAGT (300) Cy5 -TCACGCTGGGGCCTCAGTAGATTTAGCA-BHQ-2 (50)	*Culicoides* sp.[4)] (29)
C. dewulfi	dew_F dew_R dew_P	ATGCCGGAGCCTCG (300) GGGTATTTGTTCAAATAATATTCTATTTGGT (300) Cy5 -CCTGCATTTGGCAGGAATTAGCTCAATCC-BHQ-2 (50)	None
C. grisescens G1 + G2	gri_F gri_R gri_P	CMYTWCATYTWGCAGGTATYTCTTCA (300) CTAARACTGGRAGRGAWARAAGTAAAAG (900) FAM -TGGAATTACATTTGATCGAATAC-NFQ-MGB (200)	*Culicoides* sp.[4)] (30)
C. imicola	imi_F imi_R imi_P	TCCTCGAATAAATAATATAAGTTTTTGAATATTA (300) ACATTTGCYGATAATGGAGGA (300) FAM -CCATCTATTACTCTTCTTTTATTAAG-NFQ-MGB (50)	None
C. lupicaris	lup_F lup_R lup_P	AATTTCTTCTATTCTAGGAGCTGTG (300) GCCAAAACTGGTAAAGAAAGTAATAAT (300) ROX- ATGCGATCTAATGGAATTTCATTCGACCGTATACC-BHQ-2 (100)	None
C. obsoletus O1	obs1_F obs1_R obs1_P	GAAAAYGGAGCAGGAACC (50) GAAAAAATAGCCAAATCTACAGAA (300) VIC -TGCATGAGAGATATTAGATGAAAGG-NFQ-MGB (100)	*C. scoticus*[4)] (29)
C. obsoletus O2	obs2_F obs2_R obs2_P	GGAGCCGTTAATTTTATTACAACC (50) CTGCTAATACAGGTAAAGATAGTAGG (50) FAM-TGGAATAACTTTCGATCGAATACCTTTATTTGTCTGATCAGT-BHQ-1 (100)	*C. scoticus* (27/28), *C. chiopterus*[4)] (30)
C. pulicaris	pul_F pul_R pul_P	CGGAATCTCATTTGACCGTATG (300) AATGTTTCGATCAGTTAAAAGTATYGTG (300) Cy5 -ACTGTTACTCTCTCTTCCCGTATTAGCCGGAGC-BHQ-2 (100)	None
C. scoticus	sco_F sco_R sco_P	CCCCCACTYTCAGCA (300) GCTAATACCTGCTAAATGYAGA (300) ROX -TGTCTCCCATGCAGGAGCCTCAGTTGA-BHQ-2 (50)	*C. chiopterus*[4)] (26)
Culicoides sp.	Csp_F Csp_R Csp_P	AACGGAGCCGGTACC (300) YCCGAGAATTGAAGAAATACCG (300) HEX-TGCCAATATTTCTCATGCCGGAGCATCTGTAGATTTA-BHQ-1 (100)	None

2

[1)] Ct value ≤ 30 cycles when tested with 5 x 10^9 cloned targets. No cross-reactivity (i.e. Ct value ≤ 40 cycles) at all was observed when testing 5 x 10^6 targets. For comparison: Ct values with proper sequences were 8.5-10.5 (5 x 10^9 targets) and 18.6-20 (5 x 10^6 targets).

[2)] Species and numbers of haplotypes tested: *C. chiopterus*: 3; *C. circumscriptus*: 2; *C. comosioculatus*: 1; *C. deltus*: 2; *C. dewulfi*: 1; *C. fascipennis*: 1; *C. festivipennis*: 1; *C. grisescens* G1: 2; *C. grisescens* G2: 1; *C. imicola*: 1; *C. lupicaris*: 1; *C. obsoletus* O1: 4; *C. obsoletus* O2: 3; *C. pallidicornis*: 1; *C. pulicaris*: 2; *C. punctatus*: 2; *C. scoticus*: 3; *Culicoides* sp.: 4; one haplotype each of other Ceratopogonidae: *Atrichopogon* sp.; *Brachypogon* sp.; *Dasyhelea* sp.; *Forcipomyia* sp.; *Serromyia* sp.

[3)] MGB: minor groove binding probe.

[4)] Only one haplotype of the indicated *Culicoides* species positive; for number of haplotypes tested per species see footnote 2 above.

Figure

Fig. 1A

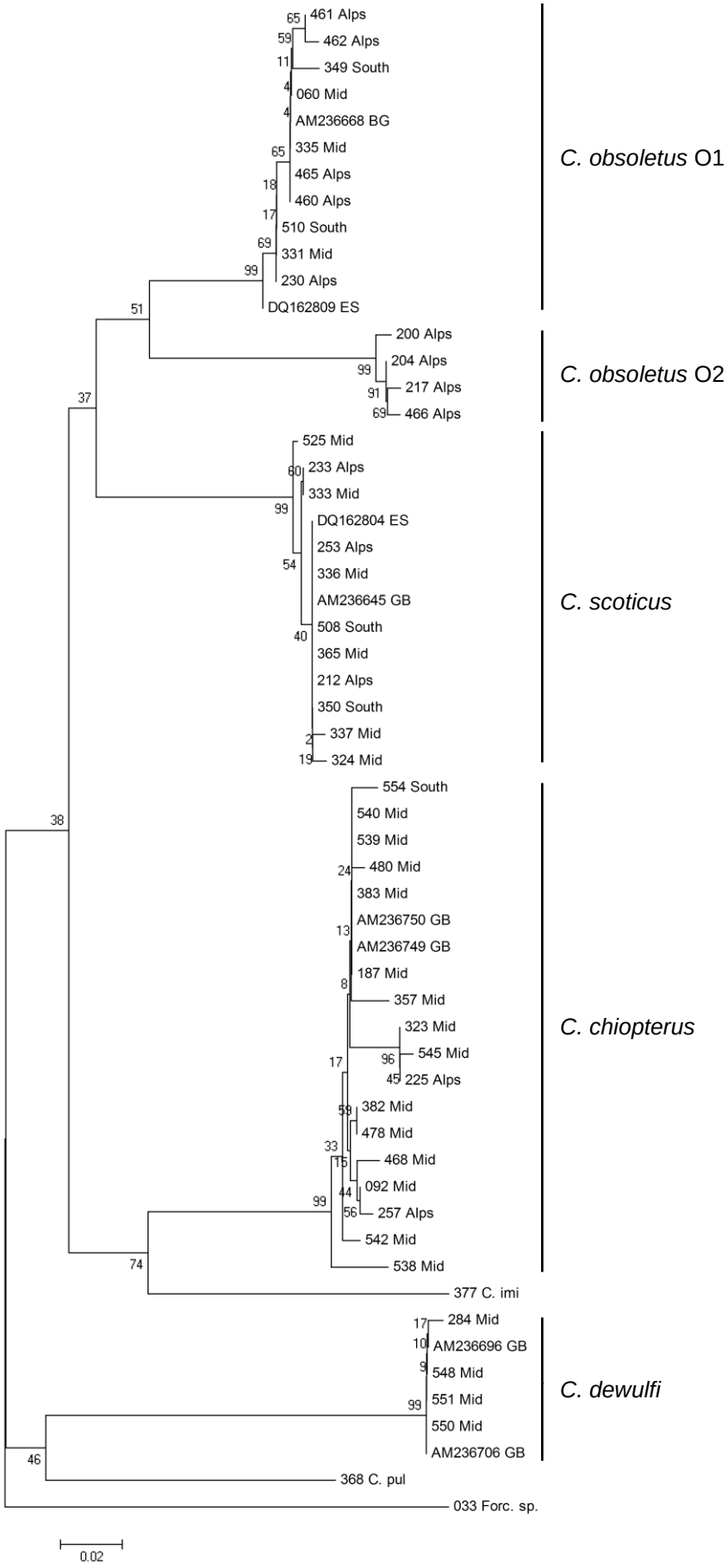
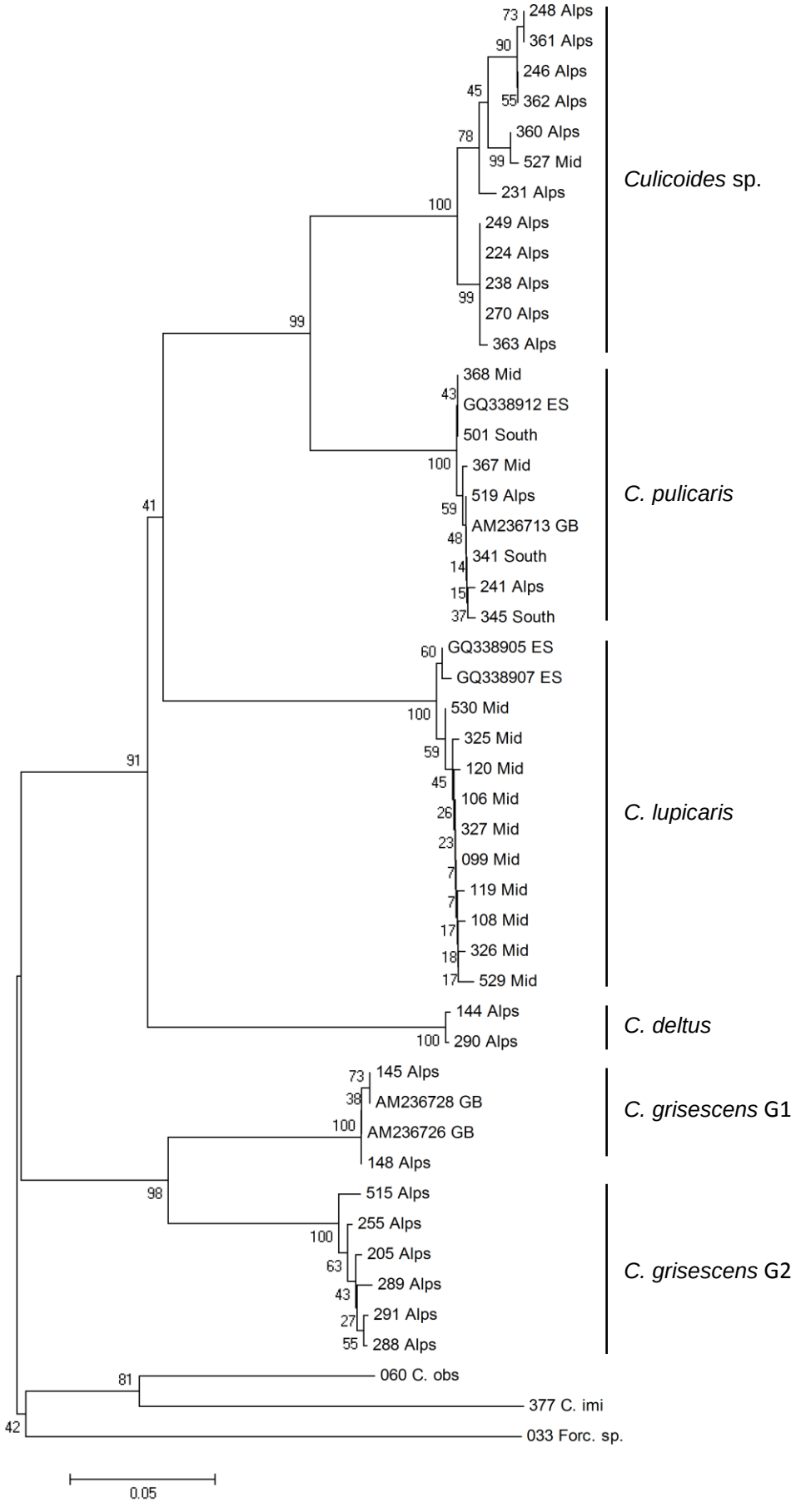


Fig. 1B


248 Alps
361 Alps
246 Alps
362 Alps
360 Alps
527 Mid
231 Alps
249 Alps
224 Alps
238 Alps
270 Alps
363 Alps
Culicoides sp.
368 Mid
GQ338912 ES
501 South
367 Mid
519 Alps
AM236713 GB
341 South
241 Alps
345 South
C. pulicaris
GQ338905 ES
GQ338907 ES
530 Mid
325 Mid
120 Mid
106 Mid
327 Mid
099 Mid
119 Mid
108 Mid
326 Mid
529 Mid
C. lupicaris
144 Alps
290 Alps
C. deltus
145 Alps
AM236728 GB
AM236726 GB
148 Alps
C. grisescens G1
515 Alps
255 Alps
205 Alps
289 Alps
291 Alps
288 Alps
C. grisescens G2
060 C. obs
377 C. imi
033 Forc. sp.
0.05