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Novel Method for Identifying the Culicoides (Diptera: Ceratopogonidae) Larvae Stage IV

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Abstract

Background: Many entomological investigations of Culicoides have used the adult specimens as samples for their studies. This is because immature stages are poorly known in most Culicoides species. The lack of correct identification systems of Culicoides larvae has been one of the major obstacles to understanding the field ecology of Culicoides species.

Materials and methods: Culicoides larvae stage was extracted from mud. Larvae stage 2 and 3 were placed in Petri dishes to continue their development to stage IV after pupation.

Larvae stage IV showing the same morphology was divided into 2 groups. First group was grown up to reach the adult stage. Second group of larvae stage IV was used for molecular identification. Thus, DNA was extracted and used as a template for a genus-specific PCR. PCR products were then sequenced and analyzed.

Results: This seems to be the first report identifying Culicoides larvae stage IV by molecular and morphological techniques. By using this method, the sequence of Internal Transcribed Spacer 1 of two Culicoides larvae: *C. circumscriptus* and *C. Puncticollis* are identified for the first time.

Keywords: Culicoides species; Diptera, Larvae stage IV; Morphological identification; Molecular detection; Tunisia

Introduction

Culicoides biting midges are the biological vectors of a range of important arboviruses of livestock, including bluetongue virus (BTV) and African horse sickness virus (AHSV) [1]. In the Mediterranean basin as well as sub-Saharan Africa, the main vector of BTV and AHSV that cause devastating diseases in ovine and equidae respectively, is *Culicoides imicola* [2]. Nevertheless, other Palaearctic Culicoides species, mainly within the subgenera Avaritia and Culicoides, such as *C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. pulicaris* are either proved or potential BTV vectors [3-9]. In spite of their economic, medical and veterinary importance, the preimaginal stages of Culicoides larvae, have received little attention than that of adults [10].

Indeed, vast majority of Culicoides species are recognized from adult stage only [11] and are sorted by the spotted wing pattern and fine structures of antenna, palpus and male genitalia. However, it is extremely difficult to distinguish the Culicoides larvae by morphological features. It might be also due to the difficulty in collecting immature stages compared to adults.

These problems have been major obstacles to understanding the field ecology of Culicoides species [12].

In spite of all the above mentioned obstacles, having more information on larval instars becomes a point of paramount importance for several reasons. On the one hand, and from the ecological side, the numbers of larvae surpass the number of adults. Thus, the identification of larval instars is essential to predict the role of species in the environment [13,14]. On the other hand, knowledge of the larval taxonomy provides clues about their diet and feeding habitats that cannot be obtained from the adult morphology [15,16].

However, many studies were carried out to identify the insect larvae species. These methods have been used until now, but each of them has certain point's limits [17].

Firstly, to identify the field-collected larvae, reliable identification key were used from other regions. However, this method can misidentify the larvae and the larval instar cannot be determined [18,19]. Secondly, other methods choose to identify species by rearing field-collected larvae before they grow to adult stage. The larval description is detailed based on photographs and exuvia(e) which makes these descriptions really difficult. Certainly, when different specimens are used for species identification and description, mistakes can supervene due to confusion between species. Other method implicates using larval specimens of all instars from eggs obtained from adults. This technique is trustworthy but often laborious and hard work. Moreover, it cannot be used to identify species for which rearing procedures are not scheduled [20].

Recent studies have used a new method for the identification of the Culicoides species at the larvae stage. This technique is based on the comparison of DNA sequences from unidentified larval samples with those of identified adult samples. In general, it can correctly identify all larval specimens to species level [21]. This method, called "DNA

barcoding", has revealed previously unknown morphological and ecological traits of many species during larval stages [22,23]. Moreover, molecular diagnostic methods have many limitations [24] including the inability to determine the instars of larvae.

To address this issue, the current study combines morphological and molecular approaches for the identification of Culicoides larvae in order to accumulate information concerning their field ecology, especially larval habitats.

Materials and Methods

Collection of larvae

Mud samples were collected from: wadi El Maleh and Bir Zira which are located on the Tunisian coast (Figure 1). 750 cm^3 sized mud samples were scraped from the soil surface using a flat trowel parallel line to the water's edge. Samples were carried to the laboratory and placed in crystallizers closed by a glass plate to prevent any risk of losing adults.

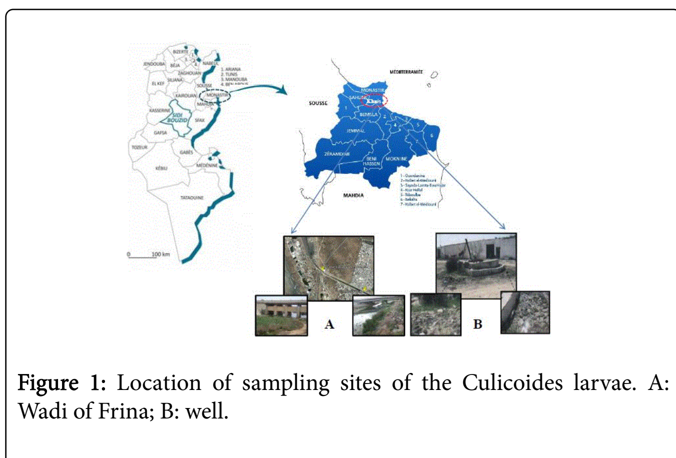

Figure 1: Location of sampling sites of the Culicoides larvae. A: Wadi of Frina; B: well.

Larvae were extracted from mud samples by using a floatation technique with magnesium sulfate [25]. Culicoides larvae stage IV was separated from other (L2 and L3) stages. These two stages (L2 and L3) continue their development in Petri dishes. Stage IV larvae with similar morphology are used for morphological and molecular identification (Figure 2).

DNA extraction

Template DNA was extracted from individual Culicoides larvae stage IV using the ZR Insect and Tissue DNA Kit_5TM (Zymo Research, USA) according to the manufacturer's instructions. The DNA was eluted in a final volume of 100 µL of the supplied elution buffer.

PCR and sequencing analysis

A locus of the Internal Transcribed Spacer 1 (ITS1) gene was amplified using the primers PanCulF (5'-GTAGGTGAACCTGCGGAAGG-3') and PanCulR (5'-TGCGGTCTTCATCGACCCAT-3') [26].

Amplification conditions were just the same as proposed by the latter authors: an initial denaturation stage at 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 1 min; annealing 58°C for 1 min and extension at 72°C for 1 min and a final extension phase at 72°C for 10 min by using Taq polymerase (QIAGEN GmbH).

Amplicons were examined by electrophoresis in 1.5% agarose gel stained with ethidium bromide. PCR products were purified and sequenced using the same primers as those for PCR (Eurofins MWG Operon, Munich, Germany).

Sequences were edited using the Chromas software version 2.33 (http://www.technelysium.com.au/chromas.html) and identified by comparison with the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST).

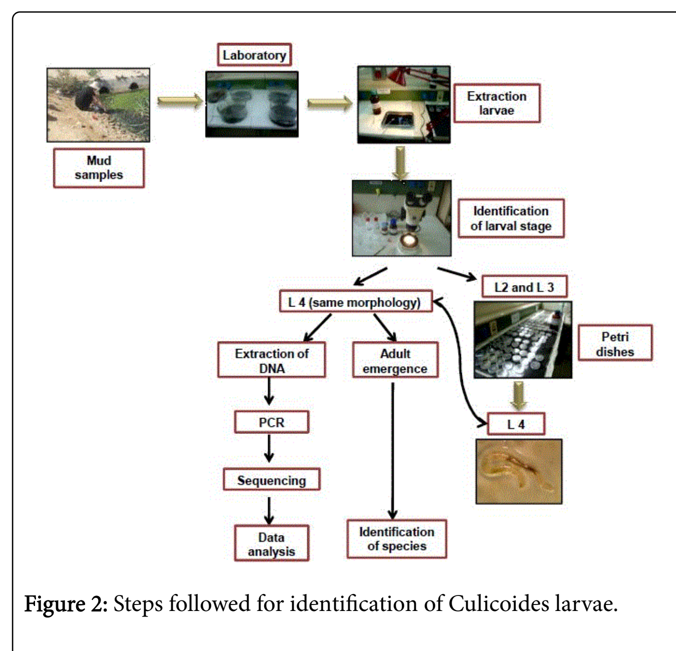

Figure 2: Steps followed for identification of Culicoides larvae.

Results and Discussion

Throughout the study, 1340 larvae were extracted from mud samples. These larvae were divided into two groups. First group, contains Culicoides larvae stage 2 and 3 (n=893). This group was placed at Petri dishes to continue their growth to stage IV. Whereas the second group, contains the larvae stage IV with same morphology (n=447), which was later divided also into two other groups. One group was designed for the DNA extraction (n=50) and the other (n=397) was used for the emergence to adult stage. Morphological identification of the emerged adult (397) showed that these larvae stage IV belonged to the following species: *Culicoides circumscriptus* (n=200) and *Culicoides puncticollis* (n=197). For the molecular identification, larva stage IV of each Culicoides species collected was selected (four specimen of each group with the same morphology). PCR using the mentioned primer set successfully produced a band of the predicted size of 490 bp (Figure 3) from larvae stage IV. PCR products were sequenced, edited and compared with GenBank database and then identified to the species level.

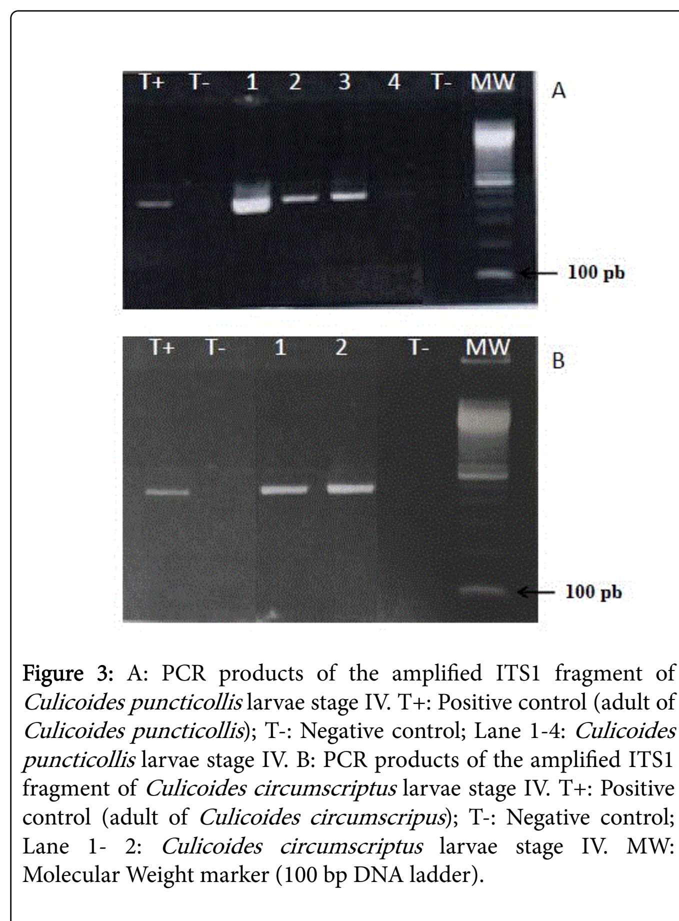

Figure 3: A: PCR products of the amplified ITS1 fragment of *Culicoides puncticollis* larvae stage IV. T+: Positive control (adult of *Culicoides puncticollis*); T-: Negative control; Lane 1-4: *Culicoides puncticollis* larvae stage IV. B: PCR products of the amplified ITS1 fragment of *Culicoides circumscriptus* larvae stage IV. T+: Positive control (adult of *Culicoides circumscripus*); T-: Negative control; Lane 1- 2: *Culicoides circumscriptus* larvae stage IV. MW: Molecular Weight marker (100 bp DNA ladder).

Sequence analysis revealed 98-99% similarity between the obtained and the GenBank deposited ones. Among, the identified species the majority corresponds to *Culicoides puncticollis* and *Culicoides circumscriptus* (Table 1).

Culicoides species	Accesion numbers of deposited sequences	Number of tested larvae	Nucleotide identities (%)
Culicoides puncticollis	AY861158.1	4	98
Culicoides circumscriptus	AY861163.1	4	99

Table 1: Nucleotide sequence identities between the larvae stage IV and deposited sequences.

The molecular identification by the using the ITS1 PCR-sequencing method meets with that by morphological identification.

In this study, the identification of Culicoides species at larva stage by molecular technique are presented for the first time. To search larval habitats, many techniques have been used. Indeed, some studies brought back the samples, such as mud and animal dung, to laboratories and kept them for several weeks until adult midges emerged [27-30]. Furthermore, only 13% and 17% of species are known as larvae and pupae respectively [31]. This may be due to the relative difficulty in collecting immature stages compared to adults. Thus, combined morphological and molecular identification of Culicoides larvae were a prerequisite to accumulate information concerning their field ecology, especially larval habitat.

This preliminary finding raises the speculation and highlights the involvement of molecular method for the identification of Culicoides larvae instars IV. The biotope of the larvae of the two species collected in this study, *Culicoides circumscriptus* and *Culicoides puncticollis* has been identified and its morphological features were described [25]. This study, however shows that the nucleotide difference between the larval and deposited sequences correspond to adult stage were maximally 1-2% in *Culicoides circumscriptus* and *Culicoides puncticollis*. The results corroborate the findings in previous reports of molecular identification of Culicoides larvae which showed that the nucleotide differences between the larval and deposited sequences were maximally 3-4% in *Culicoides kibunensis* and *Culicoides verbosus* [13].

By using this method, it is now possible to identify both the larvae stage and its corresponding species. The method has been a valuable tool to predict the adult emerging time and consequently to recommend appropriate control measures. Besides, details on the biology are essential to identify key factors limiting pest population.

Supporting Information

S1 Table: Complete nucleotide sequence for both larvae species: *Culicoides circumscriptus* and *Culicoides puncticollis*

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Conflicts of Interest Statement

The authors have no conflicts of interest concerning the work reported in this paper.

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