


## Research Article

# Xenomonitoring of Mosquitoes (Diptera: Culicidae) for the Presence of Filarioid Helminths in Eastern Austria

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Information on mosquito-borne filarioid helminths in Austria is scarce, but recent discoveries of *Dirofilaria repens* indicate autochthonous distribution of this parasite in Eastern Austria. In the current xenomonitoring study, more than 48,000 mosquitoes were collected in Eastern Austria between 2013 and 2015, using different sampling techniques and storage conditions, and were analysed in pools with molecular tools for the presence of filarioid helminth DNA. Overall, DNA of *D. repens*, *Setaria tundra*, and two unknown filarioid helminths were documented in twenty mosquito pools within the mitochondrial *cox1* gene (barcode region). These results indicate that *S. tundra*, with roe deer as definite hosts, is common in Eastern Austria, with most occurrences in floodplain mosquitoes (e.g., *Aedes vexans*). Moreover, DNA of *D. repens* was found in an *Anopheles plumbeus* mosquito close to the Slovakian border, indicating that *D. repens* is endemic in low prevalence in Eastern Austria. This study shows that xenomonitoring is an adequate tool to analyse the presence of filarioid helminths, but results are influenced by mosquito sampling techniques, storage conditions, and molecular protocols.

## 1. Introduction

In Europe, filarioid helminths of veterinary and/or medical relevance have mainly been documented in Mediterranean regions, but increasingly these pathogens are being reported in temperate climate zones in Central and Northern Europe as well [1–3].

The most important filarioid helminths in Europe are *Dirofilaria immitis* and *D. repens*, causing canine pulmonary (*D. immitis*), subcutaneous (*D. repens*), and ocular (mainly *D. repens*) dirofilariosis [4]. Both, *D. immitis* and *D. repens*, are zoonotic parasites [4]. The first Central European discoveries of *D. immitis* were confirmed in Switzerland, in

1995 and 1998 [5, 6]. Since then, both parasites, *D. immitis* and *D. repens*, have been described in humans (accidental hosts), dogs (definite hosts), and mosquitoes (vectors) in many Central European countries. Both filarioid species have now been shown to be present in all countries neighbouring Austria except for Liechtenstein, namely, Switzerland, Italy, Slovenia, Hungary, Slovakia, the Czech Republic (*D. repens* only), and Germany [1, 3, 4, 7–11]. *D. repens* was documented in most Central European countries prior to *D. immitis*.

Mosquito-borne filarioid helminths of the genus *Setaria* mainly parasitize in the abdominal cavities of artiodactyls, hyracooids, and equines. Mosquitoes of the genus *Aedes* are thought to be the main vectors of these parasites

TABLE 1: Number of mosquitoes and species collected in Eastern Austria included within this study.

| Mosquito species                                    | 2013 HC     | 2014 HC     | 2014 Biodiversa | 2015 Biodiversa | Total        |
|---|-------------|-------------|-----------------|-----------------|--------------|
| <i>Aedes cinereus/geminus</i>                       | 6           | 6           | 299             | 33              | 344          |
| <i>Aedes vexans</i>                                 | 1718        | 1847        | 4417            | 1179            | 9161         |
| <i>Anopheles algeriensis</i>                        | 4           | —           | —               | 3               | 7            |
| <i>Anopheles claviger</i>                           | —           | —           | —               | 13              | 13           |
| <i>Anopheles hyrcanus</i>                           | 145         | 63          | —               | 241             | 449          |
| <i>Anopheles maculipennis</i> complex               | 14          | 2           | 13              | 41              | 70           |
| <i>Anopheles plumbeus</i>                           | 9           | 26          | 150             | 196             | 381          |
| <i>Coquillettidia richiardii</i>                    | 2169        | 4333        | 1287            | 8034            | 15823        |
| <i>Culex martinii</i>                               | —           | —           | 66              | 996             | 1062         |
| <i>Culex modestus</i>                               | 31          | 8           | —               | 61              | 100          |
| <i>Culex pipiens</i> complex/ <i>Cx. torrentium</i> | 2707        | 2090        | 2118            | 7124            | 14039        |
| <i>Culex territans</i>                              | —           | 8           | —               | —               | 8            |
| <i>Culiseta annulata</i>                            | 4           | 7           | —               | 33              | 44           |
| <i>Ochlerotatus cantans</i>                         | —           | —           | 1               | 1               | 2            |
| <i>Ochlerotatus caspius</i>                         | —           | 51          | 17              | 4               | 72           |
| <i>Ochlerotatus cataphyla</i>                       | —           | —           | 7               | 5               | 12           |
| <i>Ochlerotatus communis</i>                        | —           | —           | 22              | —               | 22           |
| <i>Ochlerotatus flavescens</i>                      | —           | —           | 1               | —               | 1            |
| <i>Ochlerotatus geniculatus</i>                     | 4           | 41          | 20              | 6               | 71           |
| <i>Ochlerotatus intrudens</i>                       | —           | —           | 24              | —               | 24           |
| <i>Ochlerotatus japonicus japonicus</i>             | —           | —           | —               | 6               | 6            |
| <i>Ochlerotatus leucomelas</i>                      | —           | —           | —               | 1               | 1            |
| <i>Ochlerotatus rusticus</i>                        | —           | —           | 4               | —               | 4            |
| <i>Ochlerotatus sticticus</i>                       | 559         | 202         | 1113            | 499             | 2373         |
| <i>Uranotaenia unguiculata</i>                      | —           | —           | —               | 10              | 10           |
| <i>Aedes/Ochlerotatus</i> sp.                       | a           | a           | 735             | 217             | 952          |
| <i>Anopheles</i> sp.                                | a           | a           | 22              | 387             | 409          |
| <i>Culex</i> sp.                                    | a           | a           | 131             | 257             | 388          |
| <b>Total</b>  | <b>7370</b> | <b>8684</b> | <b>10447</b>    | <b>19347</b>    | <b>45848</b> |

HC, Hook Company Traps; Biodiversa, combination of various traps including BG-Sentinel Traps, HC, and aspirators; <sup>a</sup>mosquitoes specified to genus level were not included.

(e.g., *Ae. vexans* for *S. labiatopapillosa*) [12, 13]. *Setaria tundra* is a parasite of roe deer documented in several European countries such as Austria, Switzerland, Germany, France, Italy, Hungary, Poland, Spain, and Denmark (summarized in Enemark et al. [14]). In Northern Europe, *S. tundra* can also be found in domestic reindeer, wild forest reindeer, and moose [15]. This species is associated with climate changes and causes severe outbreaks of periodontitis in semidomestic reindeer in Finland [16].

The aim of this study was to xenomonitor Eastern Austrian mosquitoes for the presence of DNA of filarioid helminths—with the main focus on *Dirofilaria repens* and *D. immitis* but also on *Setaria tundra* and other mosquito-borne filarioid helminths.

## 2. Materials and Methods

The present study combines the analysis for filarioid helminth DNA in mosquitoes sampled in two independent mosquito monitoring programs using two different storage conditions (dry and  $-80^{\circ}\text{C}$ ) conducted between 2013 and 2015.

**2.1. Mosquito Sampling Method 1.** In 2013 and 2014, adult female mosquitoes were trapped at three locations in Vienna using new standard miniature light traps (John W. Hook Company, Gainesville, Florida) baited with  $\text{CO}_2$ . Collection

was carried out on a daily basis for 24 hours from March to October. Mosquitoes were killed using the insecticide dichlorvos as soon as they entered the trap. Once a week, the traps were emptied, and Culicidae were dried and stored at room temperature until further processing [17].

**2.2. Mosquito Sampling Method 2.** Mosquitoes were monitored across three provinces of Eastern Austria (Burgenland, Lower Austria, and Vienna) at 35 permanent and 23 non-permanent trapping sites. At permanent sampling sites, mosquitoes were monitored on a regular basis every second week for a 24-hour time period from April to October 2014–2015 using Biogents Sentinel Traps (Regensburg, Germany) equipped with carbon dioxide as attractant. Nonpermanent sampling sites were investigated at least once and up to six times during the summer months using Biogents Sentinel Traps (Regensburg, Germany) or exhausters. All mosquitoes were stored at  $-80^{\circ}\text{C}$  until further processing [18].

Mosquitoes were identified morphologically using the identification key of Becker et al. [19] and pooled by species, collection site, and date, with a maximum number of 50 individuals per pool. To each pool, 400  $\mu\text{l}$  of DNA/RNA lysis buffer (Zymo Research Corp., USA) and two ceramic beads (Precellys Ceramic Beads, Peqlab Biotechnologie GmbH) were added, and the samples were homogenized in a TissueLyser II (Qiagen, Germany). Approximately 350  $\mu\text{l}$  of

TABLE 2: Filarioid helminths in mosquitoes in Eastern Austria: Vienna, Lower Austria (LA), and Burgenland (B).

| Filarioid species         | Mosquito species                 | Collection site     | Sampling method | Collection date | Pool size | GenBank entry   | Maximum % identity to GenBank entries <sup>b</sup> |
|---------------------------|----------------------------------|---------------------|-----------------|-----------------|-----------|-----------------|--|
| <i>Dirofilaria repens</i> | <i>Anopheles plumbeus</i>        | Marchegg (LA)       | BG-Sentinel     | August 2015     | 1         | MF695085        | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes cinereus/geminus</i>    | Lobau (Vienna)      | HC <sup>a</sup> | July 2013       | 1         | MF695086        | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes vexans</i>              | Marchegg (LA)       | BG-Sentinel     | July 2014       | 25        | MF695087        | >99%   |
| <i>Setaria tundra</i>     | <i>Aedes</i> sp.                 | Lobau (Vienna)      | HC <sup>a</sup> | August 2014     | 9         | MF695088        | >99%   |
| <i>Setaria tundra</i>     | <i>Aedes vexans</i>              | Lobau (Vienna)      | HC <sup>a</sup> | August 2014     | 42        | MF695089        | 100%   |
| <i>Setaria tundra</i>     | <i>Coquillettidia richiardii</i> | Lobau (Vienna)      | HC <sup>a</sup> | August 2014     | 50        | MF695090        | >99%   |
| <i>Setaria tundra</i>     | <i>Aedes vexans</i>              | Vienna              | Aspirator       | August 2014     | 1         | MF695091        | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes vexans</i>              | Marchegg (LA)       | BG-Sentinel     | August 2014     | 3         | nd <sup>c</sup> | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes cinereus/geminus</i>    | Eckartsau (LA)      | BG-Sentinel     | June 2015       | 1         | MF695096        | >99%   |
| <i>Setaria tundra</i>     | <i>Aedes</i> sp.                 | Klosterneuburg (LA) | BG-Sentinel     | June 2015       | 25        | nd <sup>c</sup> | 100%   |
| <i>Setaria tundra</i>     | <i>Culex pipiens</i> complex     | Bruckneudorf (B)    | BG-Sentinel     | July 2015       | 50        | nd <sup>c</sup> | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes</i> sp.                 | Götzendorf (LA)     | BG-Sentinel     | July 2015       | 6         | MF695092        | 99%  |
| <i>Setaria tundra</i>     | <i>Aedes vexans</i>              | Götzendorf (LA)     | BG-Sentinel     | July 2015       | 50        | MF695093        | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes vexans</i>              | Götzendorf (LA)     | BG-Sentinel     | July 2015       | 50        | nd <sup>c</sup> | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes vexans</i>              | Jennersdorf (B)     | BG-Sentinel     | July 2015       | 1         | MF695094        | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes</i> sp.                 | Bruckneudorf (B)    | BG-Sentinel     | July 2015       | 4         | nd <sup>c</sup> | 100%   |
| <i>Setaria tundra</i>     | <i>Aedes vexans</i>              | Bruckneudorf (B)    | BG-Sentinel     | July 2015       | 26        | MF695095        | 100%   |
| <i>Setaria tundra</i>     | <i>Culex pipiens</i> complex     | Vienna              | BG-Sentinel     | August 2015     | 50        | nd <sup>c</sup> | 100%   |
| Filarioidea               | <i>Culex modestus</i>            | Vienna              | HC <sup>a</sup> | June 2014       | 1         | nd <sup>c</sup> | 93%  |
| Filarioidea               | <i>Culex pipiens</i> complex     | Vienna              | HC <sup>a</sup> | September 2014  | 1         | nd <sup>c</sup> | 95%  |

<sup>a</sup>Hook Company CO<sub>2</sub> baited mosquito traps; <sup>b</sup>analysis of maximum identity to GenBank Entries was performed on August 4, 2017; <sup>c</sup>sequences were not uploaded to GenBank (e.g., short sequences or poor sequence quality).

the homogenized pulp was loaded onto a QIAshredder (Qiagen, Germany). The filled QIAshredders were centrifuged for two minutes at 13,000 rpm to filter the samples (solid components remained on the column). In the next step, DNA was extracted using a ZR-Duet™ DNA/RNA MiniPrep kit (Zymo Research Corp., USA) according to the manufacturer's instructions.

DNA extracted from female mosquito pools was examined for the presence of genomic material of filarioid helminths using primers and PCR conditions published elsewhere [20]. The primers used target a 724 bp fragment of the mitochondrial cytochrome oxidase subunit I gene and are specific for various filarioid helminths (e.g., *Dirofilaria*, *Wuchereria*, *Brugia*, *Onchocerca*, *Setaria*, and *Acanthocheilonema*). PCR products were separated by electrophoresis in 2% agarose gels stained with Midori Green Advance DNA stain (Nippon

Genetics Europe, Germany). Finally, purified PCR products were sequenced by a commercial company (LGC Genomics GmbH, Germany). Sequences thus obtained were compared for similarity to sequences available in GenBank® database (<http://www.ncbi.nlm.nih.gov/BLAST>).

### 3. Results and Discussion

45,848 mosquitoes representing 25 mosquito species were analysed for the presence of filarioid DNA in this xenomonitoring survey (Table 1), resulting in the identification of DNA from *D. repens*, *S. tundra*, and two unknown filarioid helminths in 20 of the mosquito pools (Table 2; Figure 1).

DNA of *D. repens* was only found in 2015 in a single *Anopheles plumbeus* mosquito in Marchegg (Lower Austria) close to the Slovakian border. Although several mosquito

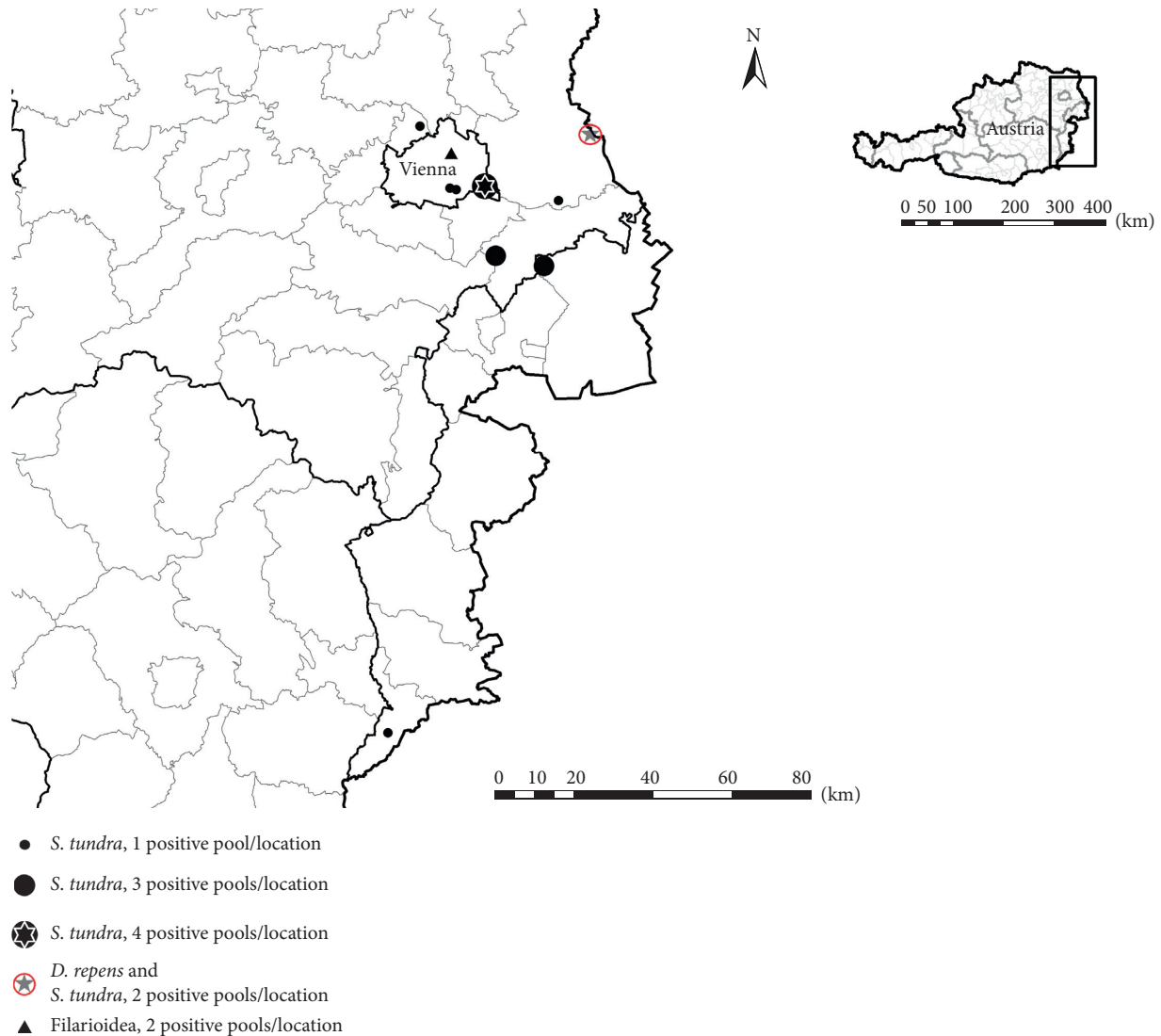


FIGURE 1: Geographic distribution of mosquito pools positive for filarioid helminths in Eastern Austria.

species of different genera are proven as potential vectors of *D. repens* [21], DNA of this parasite has so far only been detected in other *Anopheles* species (*An. algeriensis* and *An. maculipennis* complex) in Austria [2].

To date, all *D. repens* positive mosquitoes have been collected in close proximity to the Slovakian (this study) and the Hungarian borders [2]. In both Slovakia and Hungary, *D. repens* is known to be endemic with a prevalence above 10% in dogs in the Bratislava area, close to the Austrian border [22, 23]. Previous metadata analysis has shown that most reported but also potential autochthonous findings in dogs were in Eastern Austria [10]. Furthermore, Duscher et al. [24] described the examination of *D. repens* positive dogs in the same districts where positive mosquitoes were documented (Gänserndorf and Neusiedl am See). This indicates that *D. repens* might be endemic with low prevalence in this area. Simon et al. [4] postulated that two preconditions are required for a successful establishment of *D. repens* and *D. immitis* in a novel area: (i) the presence of competent mosquito vectors, which is the case in Austria,

and (ii) a certain number of positive dogs shedding microfilaria. The second precondition seems to limit the distribution of *D. repens* (but also *D. immitis*) because there are almost no stray dogs, and kennel holding is not common in Austria.

*Dirofilaria immitis* was not identified in the present large-scale survey, confirming previous results that this parasite has not yet established itself in Eastern Austria [10]. This pathogen has however been confirmed in dogs [22, 23, 25] and in mosquitoes [26, 27] in Slovakia and Hungary in the vicinity of our study area.

The most commonly found filarioid helminth within the present study area in Eastern Austria was *S. tundra*, with most occurrences of *S. tundra* DNA in mosquitoes of the genus *Aedes*, especially *Ae. vexans*. Similarly, prevalences of up to 12.3% have been reported in roe deer in Central Europe (e.g., northern Bavaria [28]). This parasite has also been recorded in *Ae. vexans* in studies in Germany and Hungary [1, 27, 29, 30], suggesting that *S. tundra* is a common parasite of roe deer in Eastern Austria.



The discovery of DNA of unknown filarioid helminths in ornithophilic *Culex* mosquitoes (*Cx. modestus* and *Cx. pipiens* complex) is not surprising because several avian filarioid helminths (with low pathogenicity for bird hosts) are present in Central Europe [29].

#### 4. Conclusions

This xenomonitoring survey confirms the presence of DNA of certain filarioid helminths in mosquitoes in Eastern Austria and indicates possible vector competence of select mosquito species. However, comparison of the two sampling techniques and storage schemes used here suggests that storage of dried mosquitoes at room temperature increases the number of false negative pools because of a decrease of DNA quality, a circumstance that has also been noticed during analyses of mosquito DNA itself [31]. Moreover, different techniques for mosquito sampling (certain mosquito species are attracted by certain traps) and the use of different PCR protocols also influence the outcome of xenomonitoring studies [32]. Nevertheless, xenomonitoring is an effective tool to examine if certain pathogens are present in an area (e.g., [27]). It can be concluded that *D. repens*, *S. tundra*, and unknown filarioid helminths (most probably avian parasites) are present in Eastern Austria. Further studies are needed to monitor in more detail the situation of *D. repens* and *D. immitis* in Austria and neighbouring countries.

#### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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