On the biology of *Simulium galeratum* in Lithuania: ecological and molecular data

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Institute of Ecology of Vilnius University, Akademijos 2, LT-08412 Vilnius, Lithuania *Simulium reptans* (L.) is a species of blackfly widely distributed in all Palaearctic. Till 2000, two species from the *S. reptans* group were recorded in Lithuania: *S. reptans* (L. 1758) and *S. galeratum* Edw., 1920.

Rubstov (1956) claimed that *S. reptans* and *S. galeratum* were different species as they differed in some morphological parameters and in biology. Edwards (1920) recognized that *S. reptans* occurs as two distinct larval morphotypes, but he did not consider them to be different species. Crosskey & Howard (2004) and other authors (Davies, 1968) regard the name *galeratum* as a synonym of *S. reptans*, so only one species of blackfly from the *Simulium reptans* group (*S. reptans* (L. 1758)) has been recorded in Lithuania (Pakalniškis et al., 2006). New molecular data on the taxonomy of *Simulium reptans* from Britain confirmed the species status of the blackfly *S. galeratum* (Day et al., 2008).

According to the majority of morphological characters (head capsule pigmentation, larval length), blackflies of the *S. reptans* group in Lithuania belong to the *S. galeratum* morphotype. The biology of *S. reptans* in Lithuania was similar to the biology of *S. galeratum* according to Rubtsov. Analysis of mitochondrial DNA COI gene sequences showed that all haplotypes from Lithuania belonged to *S. galeratum* and not to *S. reptans* blackfly species.

Key words: *Simulium reptans, galeratum*, Lithuania, mitochondrial DNA, cytochrome c oxidase subunit I (COI)

INTRODUCTION

Simulium reptans (L.) is a species of blackfly widely distributed in all Palaearctic. It is a common species in Europe, known from 32 countries (*Fauna Europea*).

In Lithuania, first data on blackflies from the *S. reptans* group were reported in 1998 (Žygutienė, Sprangauskaitė, 1998; Sprangauskaitė, 1998). Two species were recorded: *S. reptans* (L. 1758) and *S. galeratum* Edw., 1920. Blackflies of these two species developed in the largest rivers of the country (the Nemunas and the Neris) as well as in some rivers of medium size (Merkys, Vilnia). *S. galeratum* and *S. reptans* have been reported biting man and cattle in the South-Eastern part of Lithuania (Žygutienė, Sprangauskaitė, 1998; Sprangauskaitė, 1998). *S. reptans* was known to be the second pest species in Lithuania (Bernotiene, 2001).

Rubtsov (1956) claimed that *S. reptans* and *S. galeratum* were different species as they differed in larval pigmentation,

body size, details of the genitalia. According to Rubtsov, there are some differences in the biology (seasonal activity, typical habitats of development) of *S. reptans* and *S. galeratum*.

Edwards (1920) recognized that *S. reptans* occurs as two clearly distinct larval morphotypes, however, he did not consider the two morphotypes to be different species. Crosskey & Howard (2004) as well as other authors (Davies, 1968) regard the name *galeratum* as a synonym of *S. reptans*, so only one species of blackfly from the *Simulium reptans* group (*S. reptans* (L. 1758)) is thought to be known in Lithuania (Pakalniškis et al., 2006).

Molecular data are becoming a more and more important tool in the taxonomy of insects (Sebastiani et al., 2001; Szalanski et al., 2006). New molecular data on the taxonomy of *S. reptans* confirmed the species status of *S. galeratum* blackfly (Day et al., 2008).

A comparison of the first data on the region of the mitochondrial cytochrome c oxidase subunit I DNA from *S. reptans* can help to solve the question about the status of the blackfly species so economically important in Lithuania.

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MATERIALS AND METHODS

During the period 2000 to 2007, the development of *Simulium reptans* was investigated in three Lithuanian rivers (the Nemunas, the Neris and the Merkys). The material was collected from April to November at least twice per month.

Investigations were carried out in the Nemunas river (Merkinė (E24°10'46" N54°09'26"), Kulautuva (E23°39'16" N54°55'54")), the Neris river (Vilnius, E25°17'36" N54°44'42"; Kaunas, E23°54'45" N54°56'25") and the Merkys river (Merkinė (E24°10'46" N54°09'26")). Some blackflies were collected in Latvia in the Daugava river.

Blackfly larvae and pupae were collected from water plants (*Glyceria maxima* (Hartman) or *Butomus umbellatus* L.). Larvae of blackflies were counted and measured in the laboratory with a microscope (MEC-9).

Material for investigating the genetic structure was collected in 2006. Larvae and pupae were preserved in 96% ethanol at -20 °C until further analysis. DNA was extracted using a modified Robertson & MacLeod (1993) method. Blackfly pupae were put in TBE for 5 min to wash out the ethanol in which they had been stored. A single blackfly was ground between two microscope glass slides, then the homogenate was collected and incubated at 95 °C for 10 min and placed on ice for 3 min and centrifuged at 12000 g for 5 min. The supernatant with DNA was removed to a fresh tube and stored at -20 °C. The mitochondrial cytochrome c oxidase subunit I DNA was amplified with two primers specific of insects: C1-N-2191 (5'-CAG GTA AA TTA AAA TAT AAA CTT CTG G-3') and C1-J-1718 (5'-GGA GGA TTT GGA AAT TGA TTA GT-3') (Simon et al., 1994). PCR reactions were performed in a total volume of 50 μ l. Each reaction mixture contained 5 μ l of 10 \times PCR buffer, 5 µl of 25 mM MgCl2, 4 µl of 10 mM dNTP's, 4 µl of 10 pmol/ul of each primer, 1 µl of Taq Polymerase (Fermentas Inc.) and 2 µl of DNA genomic template. Ampification was carried out following the thermal cycling parameters: 96 °C for 6 min, 36 cycles of 30 s at 94 °C, 30 s at 57 °C, 90 s at 72 °C; 72 °C for 10 min. The presence or absence of PCR product was determined by running samples on agarose gel stained with ethidium bromide. Amplified DNA fragments were sequenced at the Institute of Ecology of Vilnius University. The samples were sequenced in both directions with ALFexpress II (Amersham Pharmacia Biotech AB) and using Thermo Sequenase Cy 5 Dye terminator kit (Amersham Bioscienses), following the manufacturer's protocol. The sequencing reaction consisted of 30 cycles: 30 s at 94 °C, 30 s at 55 °C and 120 s at 72 °C. When the sequences yielded unexpected results, fresh extractions were made to reanalyse the samples. Sequence confirmation was accomplished by comparing complimentary DNA strands. Editing the DNA sequences, contig assembly, and the alignment of consensus sequences were performed using the software programs ALFwin Sequence Analyser module v2.11.01 (Amersham Pharmacia Biotech AB) and Bioedit version 5.0.9. The mitochondrial COI datasets were phylogenetically analysed using MEGA version 4 (Tamura et al., 2007).

RESULTS

Investigations of the biology of Simulium reptans

Simulium reptans larvae and pupae were found abundantly in two Lithuanian rivers – the Nemunas (discharge in different segments of the river varies from 150 to 500 m³/s) and the Neris (discharge 180 m³/s). The highest density of *S. reptans* larvae was 603.3 ± 176.5 larvae / 1 dm² water plant surface in the Nemunas river (2004) and 530 ± 90 larvae / 1 dm² water plant surface in the Neris river (2005). Some larvae and pupae can be found in rivers of medium size (discharge varies from 6 to 33 m³/s) such as the Merkys (the highest density was 22.5 ± 3.5 (2004)) and the Vilnia (76 ± 29 (2004)).

Simulium reptans has two generations per year in the Nemunas and in the Neris rivers. Larvae of the first generation start their development in April–May. Water temperature at that time is usually about 10 °C. First pupae are mostly found from the beginning of May (2004, 2008) to the middle of May (2005, 2007), but also until the beginning of July. Larvae and pupae of the second generation of *S. reptans* can be found in August–September. The second generation of *S. reptans* is not abundant and in some years (2005) was even not found.

Bloodsucking activity of *S. reptans* starts in May and can last to the end of June. During investigations of the bloodsucking activity of blackflies, many *S. reptans* females were collected from dogs and cows, but only few females were collected from men.

Investigations of *Simulium reptans* morphology

The head capsule of the larvae was typical of the *Simulium* galeratum (Fig. 1) in all investigated larvae. Day et al. (2008) have found some differences between microtubercles in pupae of *S. reptans* and *S. galeratum*. Our investigations have shown that blackfly pupae collected in rivers of Lithuania have a very low density of microtubercles on the thoracic dorsum (0.45–3.5 / 25 μ m²), similarly as in *S. galeratum*, but



Fig. 1. Head capsule of Simulium reptans blackfly larva from the Nemunas river (Lithuania)

more of the microtubercles were round as was the case in *S. reptans* pupae from Britain (Day et al., 2008).

The length of blackfly larvae and the length of pupae from the Nemunas and the Neris rivers were measured. The length of *S. reptans* larvae was 4.38 ± 0.25 mm, and the length of *S. reptans* pupae was 3.01 ± 0.28 mm. According to Rubtsov (1956), the length of *S. reptans* larvae is 5.5–6 mm and of *S. galeratum* larvae 4–5 mm. The length of pupae of the two species, according to Rubtsov (1956), is 3 mm and 2.5 mm, respectively.

Nucleotide sequence variation of the COI gene in *Simulium* reptans

Of the specimens collected, a 453-bp fragment of the COI gene was sequenced from the total of seven *S. reptans* group individuals (GenBank accession GQ121362–GQ121367). Their nucleotide composition averaged over all specimens showed an A–T bias (A = 27.4%, T = 38%, C = 18.9%, G = 15.6%). A total of eight parsimony – informative characters were observed. The Dauguva and Neris-Kaunas sequences were identical and the Neris-Vilnius sequences differed from them by 2 bp. On the other hand, the Nemunas-Kulautuva, Nemunas-Merkine1 and Merkys sequences were identical, and the Nemunas-Merkine2 sequences differed from them by 1 bp (Fig. 2). These three sequences differed from the others by 9 bp.

DISCUSSION

Blackflies of the *Simulium reptans* group are very common in large rivers of Lithuania. They are also known to be a pest species (Bernotiene, 2001). The abundance of *S. reptans* larvae is decreasing in the Nemunas river due to pest control of *Simulium maculatum* blackflies as *S. reptans* and *S. maculatum* larvae develop at the same time.

According to morphological characters, blackflies of the *S. reptans* group in Lithuania belong to the *S. galeratum* morphotype. The head capsule of the larvae and larval length were typical of *S. galeratum*. The length of pupae of blackflies, collected in Lithuania, was similar to the length of *S. reptans*, so the pupae were somewhat longer than *S. galeratum* pupae should be according to Rubtsov (1956).

According to Rubtsov (1956), larvae of *S. reptans* can be found in rivers of different size, while *S. galeratum* develop only in large or medium size rivers. *Simulium reptans* produces one generation (imagos can be found in June) while *S. galeratum* has two generations per year (imagos can be found in May– June and in August). Our data have shown that *S. reptans* larvae develop in large rivers. Blackflies of the *S. reptans* group have two generations per year: in May–June and in August–September. So, according to Rubtsov, the biology of the *S. reptans* group in Lithuania is also typical of *S. galeratum*.



Fig. 2. Phylogenetic relationships of COI haplotypes (Method – neighborn joining, model Kimura 2 parameter distances, bootstrap replications 4000). Bootstrap values (>50%) are shown above the branches of clades. *Simulium morsitans* is an outgroup, EU025959 and EU025961 are COI haplotypes from GenBank described as *S. reptans* (Day et al., 2008), EU025942–EU025947, EU025949 and EU025951–EU025953 are COI haplotypes from GenBank described as *S. galeratum* (Day et al., 2008). Other names of sequences were given by the name of the rivers in which blackflies were collected

All obtained haplotypes differed from haplotypes of the same gene deposited in the GenBank; 29 sequences of the COI gene of *S. reptans* are recorded in the GenBank database (EU025942–EU025970). According to Day et al. (2008), 10 of them represent the COI gene of *S. galeratum* and others represent the COI gene of *S. reptans*. We compared our sequences with the corresponding sequences analysed in the studies of Day et al. (2008) (Fig. 2). Investigations of COI gene sequences have shown that all the specimens belonged to the *S. galeratum* species. All 22 synapomorphic positions discriminating between *S. reptans* and *S. galeratum* (Day et al., 2008) confirm the *S. galeratum* species for all specimens collected in Lithuania.

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SIMULIUM GALERATUM EDW. BIOLOGIJA LIETUVO-JE: EKOLOGINIAI IR MOLEKULINIAI DUOMENYS

Santrauka

Simulium reptans (L.) yra Palearktikoje paplitusi upinių mašalų rūšis. Iki 2000 m. Lietuvoje buvo paskelbtos dvi *Simulium reptans* rūšių grupei priklausančios upinių mašalų rūšys: *S. reptans* (L. 1758) ir *S. galeratum* Edw. (1920).

Rubtsov (1956) teigė, kad *Simulium reptans* ir *S. galeratum* – dvi savarankiškos rūšys, kurios skiriasi tarpusavyje tiek morfologiškai, tiek biologija. Edwards (1920) pripažino, kad *S. reptans* upiniai mašalai gali būti dviejų lervos stadijoje besiskiriančių morfotipų, bet jis nemanė, kad šie du morfotipai verti rūšies statuso. Šiam požiūriui pritarė Crosskey, Howard (2004) ir daugelis kitų specialistų (Davies, 1968). Jie manė, kad *S. galeratum* yra tik *S. reptans* jaunesnysis sinonimas. Todėl buvo teigiama, kad Lietuvoje randama tik viena, *Simulium reptans* grupės, upinių mašalų rūšis – *S. reptans* (L. 1758). (Pakalniškis et al., 2006). Nauji molekuliniai duomenys sudarė prielaidas teigti, kad *S. galeratum* yra savarankiška upinių mašalų rūšis, kuri skiriasi ne tik morfologiškai, bet ir mitochondrinės COI DNR sekomis (Day et al., 2008).

Lietuvoje surinktų *S. reptans* grupės upinių mašalų dauguma morfologinių požymių (galvos kapsulės pigmentacija, lervų ilgiai) tipiški *S. galeratum* rūšiai. Upinių mašalų biologija (sezoninis aktyvumas, veisimosi vietos) panašesnė į *S. galeratum* nei į *S. reptans*. Remiantis *S. reptans* rūšių grupės mitochondrinės COI DNR tyrimų rezultatais, galima tvirtinti, kad Lietuvoje gausiai aptinkama ne *S. reptans* upinių mašalų rūšis, o jai labai artima upinių mašalų rūšis – *S. galeratum* (Edw.).

Raktažodžiai: Simulium reptans, galeratum, Lietuva, mitochondrinė COI DNR