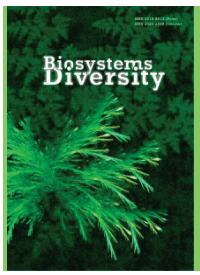

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The biology of *Simulium erythrocephalum* and *S. chelevini* (Diptera, Simuliidae): Morphological, ecological and molecular data

K. B. Sukhomlin*, M. O. Zinchenko*, O. P. Zinchenko*, V. S. Tepliuk*, Y. V. Biletskyi*, V. V. Ivantsiv*, M. G. Biletska*, L. V. Buslenko*, V. V. Ivantsiv**, S. V. Budnik*

**Lesya Ukrainka Volyn National University, Lutsk, Ukraine*
***Lutsk National Technical University, Lutsk, Ukraine*

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Lesya Ukrainka Volyn National University, Voli av., 13, Lutsk, 43025, Ukraine. Tel.: +38-033-272-01-23. E-mail: Sukhomlin.Katerina @vnu.edu.ua

Lutsk National Technical University, Lvivska st., 75, Lutsk, 43000, Ukraine. Tel.: + 38-033-274-61-03. E-mail: v.ivantsiv71@gmail.com

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The subgenus *Boophthora* is a typical Palearctic taxon, which includes only 6 species, among them. *Simulium erythrocephalum* has a transpalearctic distribution. In Europe, Adler notes only the species *S. erythrocephalum*, and Yankovsky – two species *S.* (*Boophthora*) *erythrocephalum* and *S.* (*Boophthora*) *chelevini*. According to morphological characteristics, these species differ in their life stages. We have studied the development of *S. erythrocephalum* and *S. chelevini* from three rivers of Volyn region, Ukraine (Styr, Chornoguzka, Putylivka) from 2017 to 2019. We used the EPPO PM7 / 129 standard. Collected samples, 615-bp fragments of the COI gene were sequenced from five individuals of *S. erythrocephalum* and five individuals of *S. chelevini* and compared with four samples of *S. erythrocephalum* from the GenBank. We obtained the nucleotide sequence of *S. chelevini*. All of the *S. erythrocephalum* samples from Ukraine had 692 bases, the *S. erythrocephalum* samples from Armenia had 673 bases. *S. erythrocephalum* and *S. chelevini* did not have any intraspecific variations. These intraspecific variations were not larger than the interspecific variations. It has been proved that the populations of *S. erythrocephalum* and *S. chelevini* from medium and small rivers of Volyn do not differ in biological, behavioural and genetic characteristics. Comparison of *S. erythrocephalum* and *S. chelevini* life stages showed clear differences in 20 morphological features, which are probably manifestations of phenotypic variability. Comparison of species with data from the GenBank from Spain and Armenia on the mitochondrial cytochrome c oxidase subunit I (COI) gene confirmed the opinion that *S. erythrocephalum* and *S. chelevini* are one species. On the phylogenetic tree, the data are not grouped, there is no clear separation of the clades. Bootstrap values are 95–100%, which may indicate a significant similarity of all studied samples and the lack of isolation of individual morphotypes from Volyn, Spain and Armenia. To finally confirm the taxonomic position of these two species, additional research is needed covering more individuals from different parts of Europe and analysis of more genes.

Keywords: black fly; subgenus *Boophthora*; Ukraine; mitochondrial DNA; cytochrome c oxidase subunit I (COI); taxonomy; genetic differences; phylogenetic relationships.

Introduction

Black flies are amphibiotic two-winged insects. Immature stages (eggs, larvae and pupae) are attached to the substrate in flowing watercourses, and adults live in the terrestrial habitats. These are ectoparasites of humans, farm animals, vectors of dangerous parasitic and infectious diseases (Sukhomlin & Zinchenko, 2007). Black flies occupy a leading place among blood-sucking dipterans in natural and anthropogenic landscapes of Ukraine, so the study of Simuliidae requires a detailed investigation at the genetic and morphological levels. This work is a continuation of a series of articles on the confirmation of the taxonomic status of certain problem species using modern molecular genetics methods (Zinchenko et al., 2021).

Black flies of the subgenus *Boophthora* live in the Palaearctic. The subgenus includes only 6 species (Adler, 2021. World black flies (Diptera: Simuliidae): A comprehensive revision of the taxonomic and geographical inventory): *S. bujakovi* Rubtsov, 1940 (Siberia), *S. erythrocephalum* (De Geer, 1776) (Transpalearctic), *S. guiyangense* Chen, Liu, Yang, 2016 and *S. quattuorfile* Chen, Wu, Yang, 2010 (China), *S. makunbei* (Ono, 1977), 2010 and *S. yonagoense* Okamoto, 1958 (Japan). In Europe, Adler (2021) notes only the species *Simulium erythrocephalum*, and Yankovsky (2002) – two species *Simulium* (*Boophthora*) *erythrocephalum* and *S.* (*B.*) *chelevini* Ivashchenko, 1968. Since these species are morphologically quite clearly different, we decided to find out if there were any genetic differences. For the first time in Ukraine, the species *S.* (*B.*) *erythrocephalum* was registered by Rubtsov (1940), and *S.* (*B.*) *chelevini* by Panchenko (2004). These are multivoltine (three generations are produced annually), eurytopic black flies which have exploited every conceivable habitat from trickles to rivers. Habitats of larvae and pupae of the subgenus *Boophthora* species are large (Pripyat, Zakhidnyi Bug), medium (Styr, Stokhid, Turia, Goryn, Pivdennyi Sluch, Zherev, Teterev) and small (Konopelka, Gapa, Vyzhivka, Veselukha, Lva, Putylivka, Rita, Tsyr, Kormin, Cherevakha, Rudka, Chernyavka, Ikva, Stubla, Ustya, Zamchysko, Serehivka, Zhylzhanka, Vyrka, Yazvynka, Rudynka, Muravynka, Ubort, Uzh, Zvyzdal, Loznytsia) rivers, streams and meliorative canals (Fig. 1). These species are registered as active bloodsuckers of farm animals and humans.

According to Yankovsky (2002), Kaplich et al. (2015) *S.* (*B.*) *erythrocephalum* and *S.* (*B.*) *chelevini* in morphological characteristics are different species that differ in all life stages. Adults differ in the structure of the genitalia, colour of the legs; larvae – a pattern on the frontoclypeal apotoma, the shape of the postgenal cleft, the hypostomal teeth; pupae – by gills branching and the number of spines on the abdomen VIII tergite.

Molecular data are becoming an increasingly important tool in insect taxonomy (Simon et al., 1994; Sebastiani et al., 2001; Szalanski et al., 2006; Ruiz-Arrondo et al., 2018). Studies of COI gene sequences for *S. erythrocephalum* were implemented for black flies of Armenia (Werner & Kampen, 2012; Andrianov et al., 2015) and Spain (Ruiz-Arrondo et al., 2018). For *S. chelevini* such studies were not conducted, which determined the relevance of our work.

In the literature there is only information about the biology of the black flies subgenus *Boophthora*, in particular, the distribution is de-

scribed within Central (Knoz, 1965; Živković & Burány, 1972; Jedlička et al., 2004), Northern (Crosskey, 1990; Yankovsky, 2002; Raastad, J. E., Usova, Z. V., & Kuusela, K. (2010). Blackflies of Northern Europe (Diptera: Simuliidae), CD-ROM, ETI Bioinformatics: Amsterdam), Eastern (Niesiołowski & Bokłak, 2001, 2004; Sukhomlin & Zinchenko, 2007; Kaplich et al., 2015) Europe; population dynamics (Reidelbach & Christl, 2002), life cycle (Post, 1983), the relationship between water temperature and the beginning and duration of the life cycle (Bernotiene & Bartkeviciene, 2012), gonotrophic cycle of the species (Ham & Blanco, 1984; Ruiz-Arrondo et al., 2017), cytological map of polytene chromosomes (Chubareva & Petrova, 2008).

Particular attention was paid to the medical and veterinary value of *S. etrythrocephalum* (Jedlička & Halgoš, 1982; Bardin, 2001; Ignjatovic-Cupina et al., 2006), as in recent years these black flies have proved to be an extremely aggressive anthropophilic species (Sukhomlin et al., 2019; Vujanovic et al., 2020; Sitarz et al., 2021). Therefore, there is a need to test whether these two related species also differ genetically. Comparing our data on the structure of mitochondrial DNA of the two species with each other and with the data contained in the GenBank will help to resolve the status question of these black flies species, which have important medical and veterinary significance within the Palearctic.

Materials and methods

In the period from 2017 to 2019, we studied the development of *S. erythrocephalum* and *S. chelevini* from three rivers of Volyn region, Ukraine: the River Styr (t. Lutsk E 25°29'96" N 50°74'24 "), R. Chornoguzka (vill. Polonka E 25°29'96" N 50° 68'41"), R. Putylivka (vill. Stavok E 25°57'46" N 50°41'41") (Fig. 2 a–c). The material was collected from April to November at least twice a month.

Larvae and pupae of Simuliidae were collected from the leaves of aquatic plants (Glyceria maxima (Hartman) Holmb., 1919, *Phragmites australis* (Cav.) Trin. Ex Steud., 1841, *Butomus umbellatus* Linneus, 1753) or *Salix babylonica* Linneus, 1753, which are immersed in water. Black fly larvae were counted and measured in the laboratory using a microscope (MBS-10). In Fig. 2–7 the following abbreviations are used: a – antenna; al – anal lobe; c – circus; fa – frontoclypeal apotoma, g – gill, gc – gonocoxite; gf – genital fork; gs – gonostylus; hs – hypostoma, hsc – hooks, spine comb of the pupal abdomen, hst – hypostomal teeth, hv – hypogynial valve; mp – maxillary palp; ms – medial sclerite; mt – mandibular teeth; p – paramere; sv – sensory vesicle; vp – ventral plate.

Material for the study of genetic structure was collected in 2019. Larvae and pupae were stored in 96% ethanol at –20 °C for further analysis.

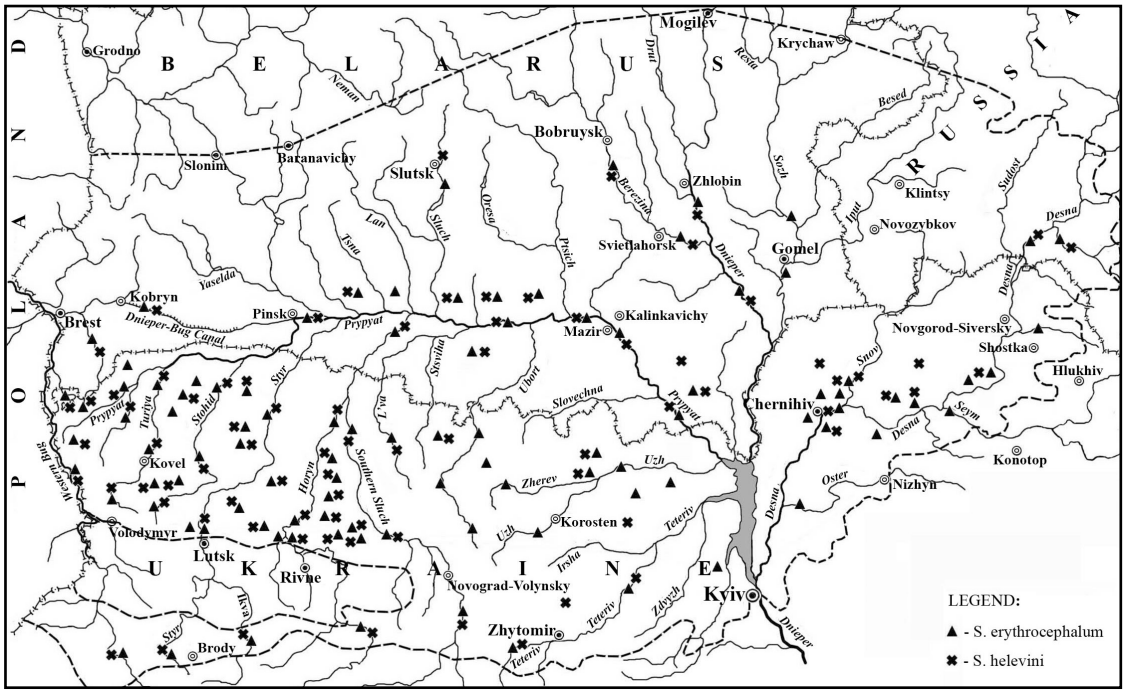

Fig. 1. Points of occurrence of *Simulium erythrocephalum* and *S. chelevini* in mixed forests of Eastern Europe

Fig. 2. Location of materials collection: *a* – Styr River; *b* – Putylivka River; *c* – Chornoguzka River

During initial processing of insect samples protocols as recommended in the EPPO PM7/129 Standard (EPPO (2016) PM 7/129 (1) DNA barcoding as an identification tool for a number of regulated pests EPPO Bulletin, 46, 501–537) was applied. Briefly, total DNA from individual larvae and pupae of different species was extracted, using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer instructions for Animal Tissue. PCR-settings to amplify the mitochondrial cytochrome c oxidase subunit I (COI) gene of insects was adapted from Folmer et al. (1994) using the primer combination LCO1490: 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and HCO2198: 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′. PCR Mastermix using 20 mg/mL BSA was prepared according to the EPPO Standard. PCR amplification was performed using the proposed PCR program with an initial denaturation at 95 °C for 2 min, followed by denaturation at 95 °C for 30 s, annealing at 49 °C for 30 s, and extension at 72 °C for 1 min, followed by 72 °C for 10 min, with a final 120 min extension step at 8 °C. To check for successful DNA amplification, the PCR products than were separated on a 1% agarose gel.

The presence or absence of PCR product was determined using a sample on an agarose gel stained with GelRed. Amplified DNA fragments were sequenced in both directions by the Sanger method using the ExoStar kit for sequencing reactions, following the manufacturer's protocol. Sequence configuration was performed by comparing free DNA strands. Editing of DNA sequences, assembly configuration and alignment of consensus sequences and phylogenetic analysis were performed using MEGA version X (Kumar et al., 2018).

Standard statistical software packages for the personal computer Statistic10 (StstSoft Inc., USA) were used for statistical data analysis. Data are presented as the mean value with standard error (x ± SE).

Results

Research into biology of S. erythrocephalum. An abundant, widespread species that exploited every habitat except spring streams flowing from swamps. Larvae and pupae live on non-silted substrates, stones and plants. The development of immature stages was observed at a water temperature of 0.5–22 °C, a dissolved oxygen content of 51–92%, and a flow velocity of 0.3–0.9 m/s. The highest density of aquatic stages (up to 1000 individuals/dm^2) was registered at a flow velocity of 0.4–0.6 m/s. Three generations are produced annually, the first generation of adults emerges in mid-May at a water temperature of 15–17 °C, the second – in late June, early July, the third – in August, early September. Eggs or larvae overwinter. Larvae overwinter in large and medium-sized watercourses, and eggs – in small ones. The difference between spring and summer forms is well traced. Spring forms are larger and brighter coloured. It is an active bloodsucker of humans and domestic animals.

Research into biology of S. chelevini. An abundant and widespread species that develops at the same time as *S. erythrocephalum*. Larvae and pupae settle on rocks, bridge piers, submerged branches of shrubs and other coastal vegetation, but prefer narrow-leaved aquatic vegetation. Eurytopic species, occurs at a water temperature of 0.4–22 °C and a flow velocity of 0.3–0.9 m/s. The density of larvae is higher in sections of rivers with a flow velocity of 0.4–0.6 m/s and a dissolved oxygen content of 55–92%. The maximum larval and pupae density (550 individuals/dm^2) was registered in the second decade of May. Three generations are produced annually. The adult emergence of the first spring generation occurs in mid-May, when the water warms up to 15–18 °C. The departure of the second generation imagoes was registered in late June, in July and the third – in late August, early September. Wintering depends on the type of watercourse. Larvae overwinter in large rivers and eggs in small rivers. There are two distinct forms: spring (large and dark) and summer (small and light). One female lays an average of 173 ± 6 eggs. Actively attacks humans and domestic animals.

Thus, the biology of the species is similar, no significant differences have been registered.

Morphological study of S. erythrocephalum. Female. Body length 3.1 ± 0.25 mm (Fig. 3). *Head.* Frons is high (length 0.17 ± 0.01 mm, width 0.16 ± 0.01 mm), with smooth edges. A few hairlike setae cover it at the edges and at the bottom. The clypeus is oval-elongated (length 0.23 ± 0.01 mm, width 0.20 ± 0.01 mm), not pubescent at the base of the antennae and with a small stripe along the clypeus. Antennae are dark brown, relatively long (0.49 mm), pedicel and flagellum 1 (0.05 mm) 1.5 times longer than the flagellum 2. The maxillary palpus is brown, the 3rd palpomere is long (0.14 ± 0.01 mm), with a small growth at the top, the 5th palpomere is shorter than the 3rd and 4th combined. Sensory vesicle is elongated (length 0.06 mm, width 0.03 mm), sensilla are arranged in groups. The mandible has 28 internal and 15 external mandibular teeth. The maxilla have 14 teeth on the maxillary lacinia and 13 on the maxillary galea. Cibarium is rectangular (length 0.20 mm, width 0.13 mm), with narrow and long (0.03 mm) inwardly curved cornua and cibarial armature.

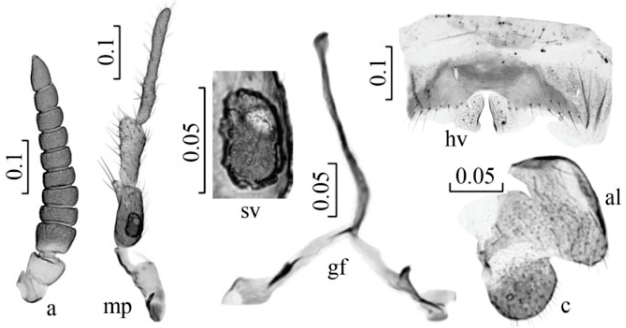

Fig. 3. Some structures of the female *S. erythrocephalum*

Thorax. The scutum is black, with sparse golden hairlike setae, the pattern is blurred, silvery spots are indistinct.

Legs. The colour is greyish-brown. The femurs and tibiae gradually darken to the top. The basitarsus of the legs front pair is conical, 1.5 times shorter than the tibia. Calcipala is small (0.015 mm), its width (0.02 mm) is slightly less than half the width of the basitarsus at the distal end. Pedisulcus occupying ⅓ the width of the tarsomere 1. The claw is simple (length 0.04 mm).

Abdomen. Genital fork with a very long stem (0.21 ± 0.01 mm) and short lateral arms (0.04 ± 0.01 mm). The lateral arms are thin, diverge at an angle of 100°, on lateral plate there is a large spine. The sternite VIII with a rectangular blackout and a notch in the middle of the posterior edge. Hypogynial valves have smooth, darkened edges, slightly diverging behind, pubescent with thin hairlike setae. Anal lobes are large, rounded (length 0.09 mm, width 0.12 mm), with an elongated outer edge. Cerci are oval (length 0.04 mm, width 0.06 mm), equal to half the width of the anal lobes.

Male. Body length 2.8–3.2 mm (Fig. 4).

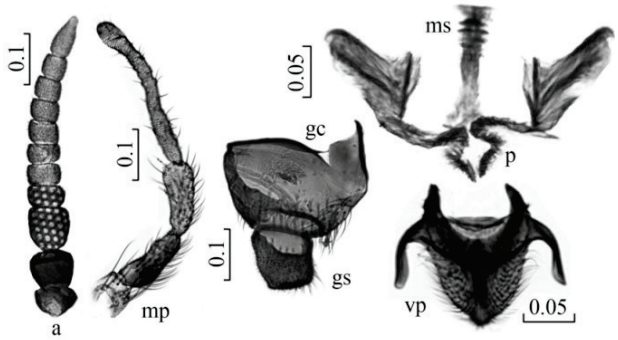

Fig. 4. Some structures of the male *S. erythrocephalum*

Head. The clypeus is oval-elongated (length 0.15 mm, width 0.12 mm), pubescent at the edges with thin small hairlike setae. Antennae relatively long (0.45 ± 0.02 mm), dark brown; the pedicel and flagellum 1 (0.05 mm) are the same length and 1.5 times larger than the flagellum 2 (0.03 mm). The maxillary palpus is short (0.47 ± 0.01 mm), brown, the 5th palpomere (0.20 mm) is equal to the length of the previous two.

Thorax. The scutum is black, velvety, covered with golden hairlike setae, the pattern is clear (butterfly-shaped). Silver spots are clear, slightly elongated along the sides. The anepisternal membrane is not pubescent Legs are dark brown. Only the tarsus hind legs in the central part are light.

The legs are more intensely coloured at the base and top. The basitarsus of the front pair of legs is slightly extended to the top, its length is about 1.5 times smaller than the tibia. Calcipala is small (length 0.015 mm), its width (0.02 mm) is 3.5 times smaller than the width of the basitarsus at the distal end. Pedisulcus occupying ⅔ the width of the tarsomere 1.

Abdomen. Ventral plate is small, lamellar (length 0.11 mm, width 0.10 mm), tapering to the top; pubescent setose lip reaches the middle of the ventral plate body; on the sides – strongly developed (length 0.07 mm) basal arms directed to the top. Gonocoxites are large, rectangular (length 0.15 mm, width 0.20 mm); lateral outgrowth is poorly expressed. Gonostylus is short (0.09 mm) and wide, almost square, with 4–5 apical spines on top. Median sclerite is short (0.095 ± 0.005 mm), strip-shaped, expanded and corrugated at the base. In the parameres there are 2–3 rows of numerous small spines, parameral subtriangular plate is large (length 0.13 mm, width 0.10 mm).

Larva. Body length 7.35 ± 0.85 mm, milky-yellow, dirty yellow colour, light head (Fig. 5).

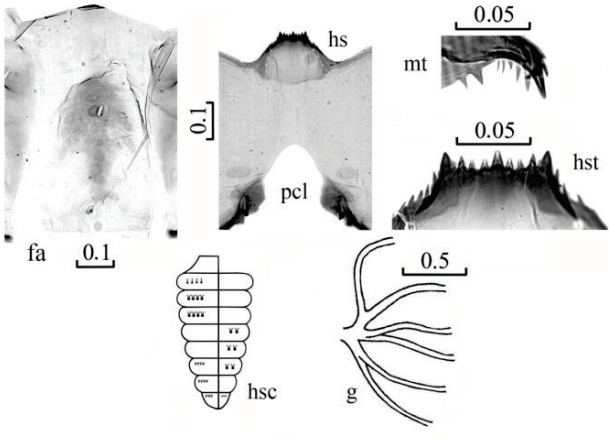

Fig. 5. Some structures of the immature stages *S. erythrocephalum*

Head. The pattern on the frontoclypeal apotoma is cross-shaped, the anteromedial head spots are indistinct, the posterolateral head spots are not pronounced. In the labral-fan are 47 ± 8 rays. Antenna is short (0.31 ± 0.01 mm), the proximal article (0.10 mm) is slightly shorter than the medial article (0.12 mm), distal article (0.09 mm) is slightly shorter than proximal article. The mandible is elongated, its length (0.29 ± 0.02 mm) is twice as great as its width (0.12 ± 0.01 mm). The mandible apical tooth is wide, wedge-shaped, directed forward, its length to the anterior pre-apical tooth is 0.01 mm; pre-apical teeth widely spaced, anterior is the largest, middle – below the level of neighbouring; 5 internal teeth; marginal teeth are sharp, wedge-shaped, do not touch each other. Maxillary palpus is long (0.12 mm), cylindrical. Hypostoma is narrowed in front (length 0.10 mm, width 0.08 mm), has 3 spiniforms on the sides. The median tooth is located above the level of the lateral ones; among the sublateral teeth the highest are the outer ones, their vertices lie almost on the same level with the lateral ones; the median tooth is the lowest. The postgenal cleft is deep (length 0.15 ± 0.02 mm, width 0.10 ± 0.01 mm), occupies half the length of the postgena.

Abdomen. Anal sclerite – with thin anal sclerite arms, front (0.13 ± 0.01 mm) shorter than the posterior (0.20 ± 0.01 mm), reaching the 11th row of hooks. In the posterior circlet are 67 ± 7 rows of hooks 14 ± 1 in each row.

Pupa. Body length 3.15 ± 0.35 mm. On III–IV tergites of the abdomen – 4 large hooks, on VII–IX – incomplete setae rows of different sizes. 6 gills: (2 + 2 + 2), the upper gill is directed upwards, bends at an angle of 90° and goes forward. The angle of divergence of the upper and lower branches at the base is approximately 180°.

Morphology study of S. chelevini. Female. Body length 3.7 ± 0.2 mm (Fig. 6). *Head.* Frons is black, shiny, high (length 0.20 ± 0.01 mm, width 0.17 ± 0.01 mm), rarely pubescent on the sides. The clypeus is elongated (length 0.27 ± 0.01 mm, width 0.23 ± 0.01 mm), pubescent on the sides, at the base is a small triangular light spot. Antennae are long (0.56 ± 0.01 mm), thick, brown, light only the scape, pedicel and base of the flagellum 1; The pedicel is equal to the length of the flagellum 1; 2nd flagellum – the widest. The maxillary palps are brown, the 3rd palpomere with a small sock on top; the 5th palpomere (0.26 mm) is longer than the 3rd and 4th combined. Sensory vesicle is oval (length 0.05 mm, width 0.035 mm), sensilla are arranged in groups. The mandible has 25 internal and 15 external teeth. Maxillary lacinia have 14 teeth and 11 – on the galea. Cibarium is elongated (length 0.22 mm, width 0.12 mm), with small (0.04 mm) curved inward cornua and setae on the upper edge.

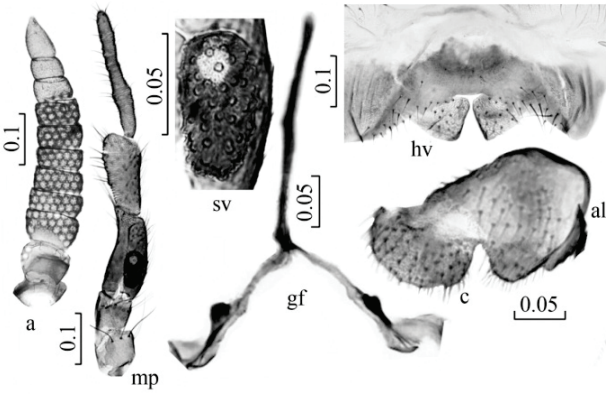

Fig. 6. Some structures of the female *S. chelevini*

Thorax. The scutum is black, shiny, pubescent with sparse and short hairs. Silver spots are indistinct. Legs are yellowish-brown. Tibia are with a small dark spot at the base and darkening at ¼ length at the top. The basitarsus of the front pair of legs is conical, its length is 1.5 times less than the tibia. Calcipala is well developed (0.02 mm), its width (0.03 mm) is ½ the width of the basitarsus at the distal end. Pedisulcus occupying ⅔ the width of the tarsomere 1. The claw is simple.

Abdomen. Fork with a thin and long (0.22 ± 0.02 mm) stem and thin high (0.12 ± 0.01 mm) lateral arms diverging at an angle of 90°, the lateral plates are not pronounced, chitinous spines-shaped thickenings are developed. The sternite VIII has a rectangular dark spot and a notch on the back edge. The inner edges of the hypogynial valves are darkened and slightly diverged, pubescent with hairlike setae. Anal lobes are large (length 0.22 mm, width 0.26 mm), almost round, elongated on the outer edge. Cerci are semicircular (length 0.06 mm, width 0.09 mm).

Male. Body length 3.0 ± 0.2 mm (Fig. 7).

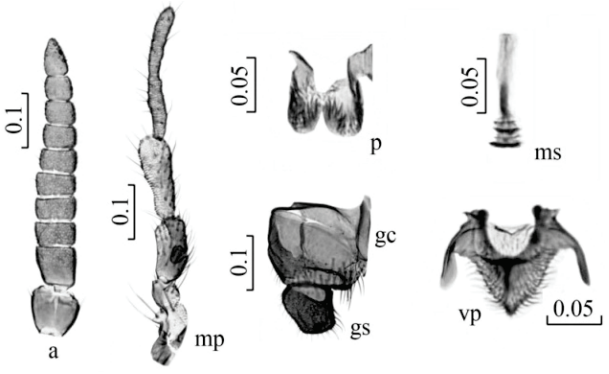

Fig. 7. Some structures of the male *S. chelevini*

Head. The clypeus is teardrop-shaped (length 0.27 ± 0.01 mm, width 0.25 ± 0.01 mm), pubescent with long hairlike setae on the sides, a small elongated light spot at the base. The antennae are dark brown, short (0.49 ± 0.01 mm), the pedicel and flagellum 1 are approximately the same length (0.06–0.07 mm) and twice as long as the 2nd (0, 03 mm), other flagellum joints are almost square. The maxillary palpus is brown, long (0.49 ± 0.01 mm), the 5th palpomere is long (0.27 mm), larger than the 3rd and 4th combined.

Thorax. The scutum is black, velvet. Silver spots are expressive. Legs: the colour is mostly dark brown. Only the central part of the basitarsus is light; tibiae are more intensely coloured on ¼ near the base and on ¼ near the top. Calcipala is well developed, its length (0.03 mm) is equal

to the width and occupies the ½ basitarsus at the distal end. Pedisulcus reaching the middle of the tarsomere 1.

Abdomen. Ventral plate is lamellar, wedge-shaped (length 0.11 ± 0.02 mm, width 0.13 ± 0.02 mm), larger than in *S. erythrocephalum*, basal arms (0.05 mm) are slightly shorter than in the previous species. Gonocoxite is large, rectangular (length 0.20 ± 0.02 mm, width 0.26 ± 0.02 mm); lateral outgrowth is underdeveloped (0.03 mm). Gonostylus almost square (0.11 x 0.12 mm), with of 5-7 apical spines on top. Median sclerite (length 0.135 ± 0.005 mm) is elongated, slightly narrowed in the central part and rounded at the top, with a transversely cut base. The parameres consist of many small spines arranged in 2–3 rows and large (length 0.15 mm, width 0.08 mm) parameral subtriangular plate.

Larva. Body length 7.5 ± 0.6 mm, grey-yellow colour with transverse brown stripes, light head (Fig. 8).

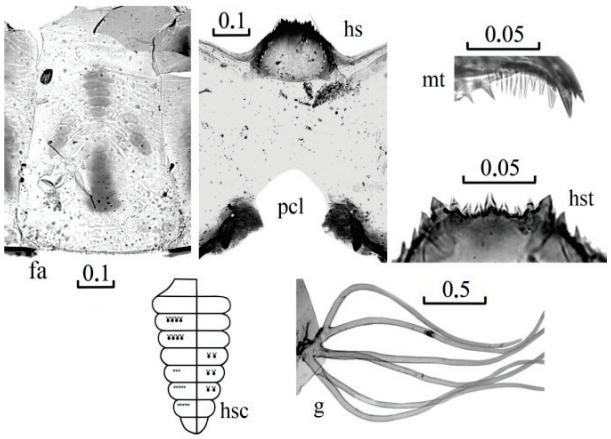

Fig. 8. Some structures of the immature stages *S. chelevini*

Head. The pattern on the frontoclypeal apotoma is clear cruciform, the posterolateral head spot are indistinct, the central ones are composed in a dark cloud. The antenna is long (0.42 ± 0.01 mm), the proximal article (0.12 mm) is equal to the distal article and slightly shorter than the medial article (0.15 mm). In the labral-fan are 41 ± 6 rays. The mandible is rectangular (length 0.34 ± 0.01 mm, width 0.16 ± 0.01 mm). The apical tooth is narrow, directed downwards, long (0.028 mm), 0.014 mm larger than the anterior pre-apical tooth; mandible pre-apical tooth widely spaced, well developed, anterior larger than median and posterior; 4 internal teeth, they are small, short (0.015 mm); 2 marginal teeth are spike-shaped. The maxillary palpus is long (0.14 mm), narrow (0.05 mm), tapering to the top. The hypostoma is narrowed forward (length 0.16 mm, width 0.08 mm). Hypostomal teeth are narrower and longer than in *S. erythrocephalum*; the tops of the median and lateral teeth lie on the same level; among the sublateral teeth the smallest is the median, inner and outer are equal in size. The postgenal cleft is rounded (length 0.19 ± 0.02 mm, width 0.18 ± 0.01 mm), occupies less than half the length of the postgena.

Abdomen. Anal sclerite with thin arms, the front arms (0.12 ± 0.01 mm) are slightly shorter than the posterior (0.17 ± 0.01 mm), reaching the 10th row of hooks. In the posterior circlet are75 ± 9 rows of hooks, 12 ± 1 in each row.

Pupa. Body length 3.35 ± 0.55 mm. On the II tergite of the abdomen are spiniforms, on III and IV – 4 large hooks, on VI–IX – 1 row of spines with different length. Gills 6: (2 + 2 + 2), the upper gill goes to the side, bends and goes forward. The angle of divergence of the upper and lower gill 130°.

Comparison of *S. erythrocephalum* and *S. chelevini* life stages showed clear differences in 20 morphological features (Table 1).

Nucleotide sequence variation of the COI gene. Collected samples of the COI gene 615-bp fragments were sequenced from five individuals (three larvae and two pupae) of *S. erythrocephalum* and five individuals (two larvae, two pupae and one female) of *S. chelevini* and compared with four samples of *S. erythrocephalum* (GenBank MG894328.1, MG894329.1, KF640027.1, KF640028.1 from Spain and Armenia). The COI sequences from the *S. erythrocephalum* did not show an A + T bias in nucleotide content (mean = 64.9%) relative to the C + G content (mean = 35.1%), as is typical of arthropods (Kumar et al., 2018; Crease, 1999). The individual average percentage of nucleotide content was: A = 27.8%, T = 37.1%, C = 17.4%, G = 17.7%. The content of thymine in the Volyn samples of *S. chelevini* is slightly lower (36.7%) than in samples of *S. erythrocephalum* from Volyn, Spain and Armenia (37.1%). The content of cytosine in the Volyn samples of *S. chelevini* is slightly higher (17.9%) than in samples of *S. erythrocephalum* from Volyn, Spain and Armenia (17.4%).

Table 1

Comparison of *S. erythrocephalum* and *S. chelevini* morphological features

No.	Morphological features	*S. erythrocephalum*	*S. chelevini*
1	Dimensions of females	small, body length 3.1 ± 0.25 mm	large, body length 3.7 ± 0.2 mm
2	The length of the 4th palpomere of the female maxillary palps	shorter than the 2nd and 3rd combined	longer than the 2nd and 3rd combined
3	The shape of the sensory vesicle	elongated	rounded
4	Lateral arms	thin and short (0.04–0.05 mm)	well developed, long (0.12–0.13 mm)
5	The angle of divergence of the lateral arms	100°	90°
6	The length of the male's maxillary palpus	short (0.47 mm), 5th palpomere (0.20 mm) is equal to the length of the previous two	long (0.49 mm), 5th palpomere (0.27 mm), larger than the 3rd and 4th combined
7	Ventral plate	relatively wide, height 2.0 times bigger than width	relatively narrow, height 1.5 times bigger than width
8	Basal arms	thin	wide
9	The length of basal arms	more than ½ body length	less than ½ body length
10	Shape of median sclerite	triangular	dumbbell
11	Median sclerite	short	long
12	Number of setae on the top of the gonostylus	4–5	5–7
13	The pattern on the frontoclypeal apotoma is positive	surrounded by a dark cloud	surrounded by a light cloud or without it
14	The shape of the postgenal cleft	trapezoidal	rounded
15	Postgenal cleft	occupies ½ the length of the postgena	takes less than ½ the length of the postgena
16	Anterior edge of the hypostoma	narrowed	expanded
17	Hypostomal teeth	thin and long	wide
18	The median tooth of the hypostoma	same level with the lateral	above the level of the lateral
19	The angle of divergence of the gills	approximately 180°	approximately 130°
20	The number of spines on VIII tergites of the abdomen	less than 4–5	above 5

Our nucleotide sequence of *S. chelevini* p1 (615 bp)

AGCCTGAGCTGGAATAGTAGGAACTTCTCTTAGAATACTTA
TTCGAGCAGAATTAGGACATCCAGGGCCTCTTATTGGAGAT
GATCAAATTTATAACGTAATTGTAACAGCACATGCCTTTGT
AATAATTTTTTTTATAGTTATACCAATTATAATTGGAGGATT
TGGTAATTGACTAGTTCCTTTAATATTAGGGGCTCCTGACA
TGGCTTTTCCCCGAATAAATAATATAAGTTTTTGAATGTTAC
CCCCCTCATTAACTCTTTTATTGGCTAGTAGTATAGTAGAA
GCAGGAGCTGGGACAGGATGAACTGTTTATCCCCCTCTATC
TTCTGGAATTGCTCATGCTGGAGCTTCAGTAGACTTAGCTA
TTTTTTCTTTACACTTAGCTGGGATTTCTTCTATTCTAGGAG
CTGTAAATTTTATTACAACCATTATTAATATACGGTCAAAT
GGAATTACTTTTGACCGAATACCTCTATTTGTATGATCAGT
AGTTATTACAGCTGTATTATTACTTCTTTCTTTACCTGTTCTT
GCCGGAGCAATTACAATACTTTTAACAGATCGAAATTTAAA
CACTTCATTTTTTGACCCTGCTGGAGGAGGAGATC.

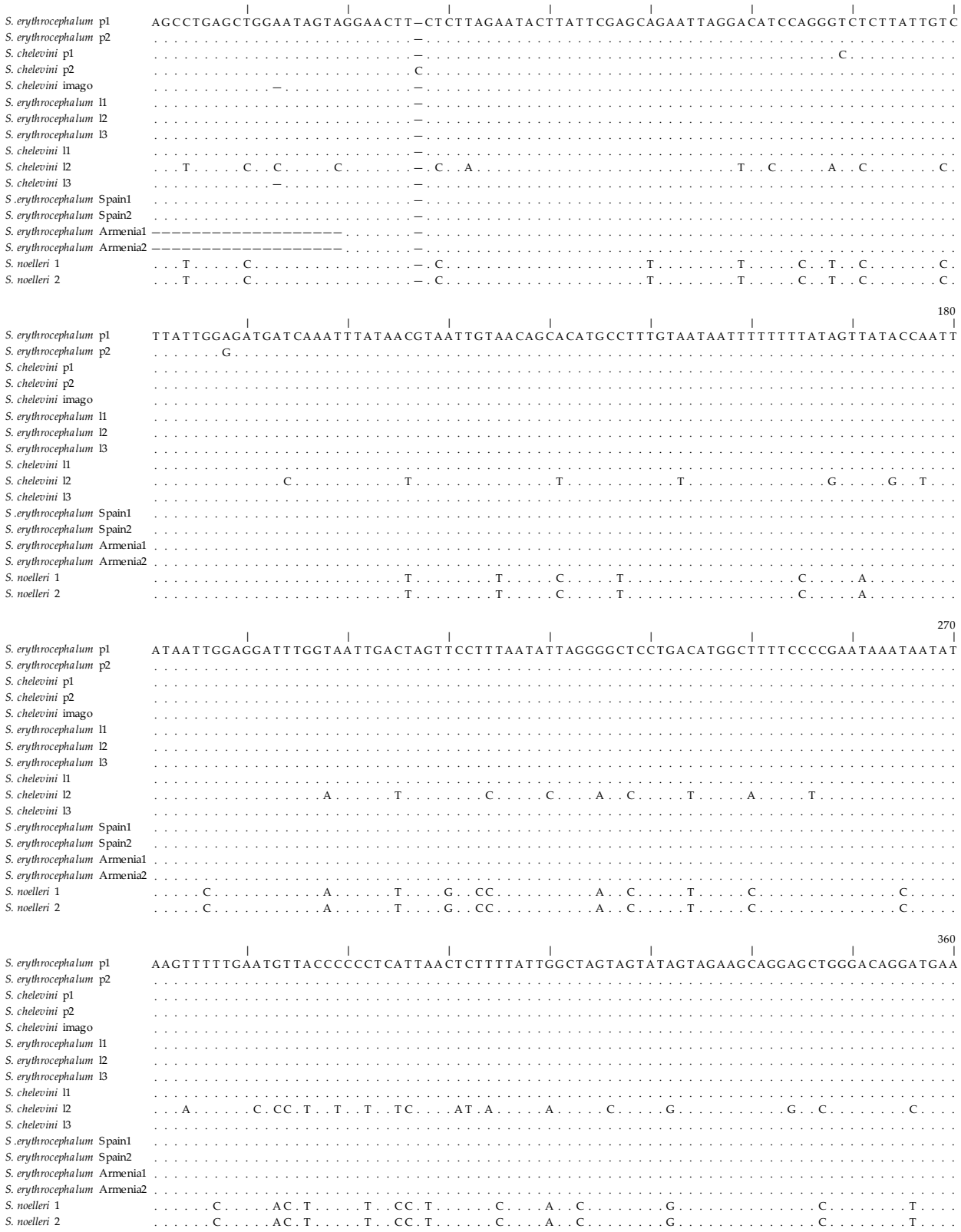

Fig. 9. Alignment of 615 bp COI gene for 17 samples, 1–360 nucleotides: a period indicates the site identical to *S. erythrocephalum* (Ukraine); a dash indicates a gap site; *p* – pupa, *l* – larvae, *1*, *2*, *3* – numbers of samples

Discussion

The biology of *S. erythrocephalum* and *S. chelevini* is similar because they are eurytopic species that have exploited every conceivable habitat from trickles to rivers. They inhabit all underwater substrates in areas where the flow velocity reaches 0.3–0.9 m/s, the content of dissolved oxygen in water is 51–92%, and development takes place at a water temperature of +0.5…+22 °C (Sukhomlin & Zinchenko, 2007). Three generations are produced annually, the development of which overlaps and the emergence is vague: emergence of adults of the first spring generation occurs in mid-May, emergence of adults of the second generation is registered in late June, early July and third – in late August, early September. Eggs or larvae overwinter (Kaplich et al., 2015).

A comparative morphological analysis of the Volyn population of *S. chelevini* with the description given by Ivashchenko (1968) revealed some differences in the structure of larvae and pupae. In larvae, the post-

genal cleft of the head capsule is arched, rounded, occupies half the length of the postgena. Armature on the pupal abdomen begins on the VII tergit. On VII–VIII – full rows of spines of different sizes, on IX – small spines. Sometimes individual spines were on the VI tergit.

Comparison of the larvae of the *S. erythrocephalum* Volyn population with the forms described by Rubtsov (1956), proved that the individuals we collected are generally similar, but have a smaller postgenal cleft of the head capsule.

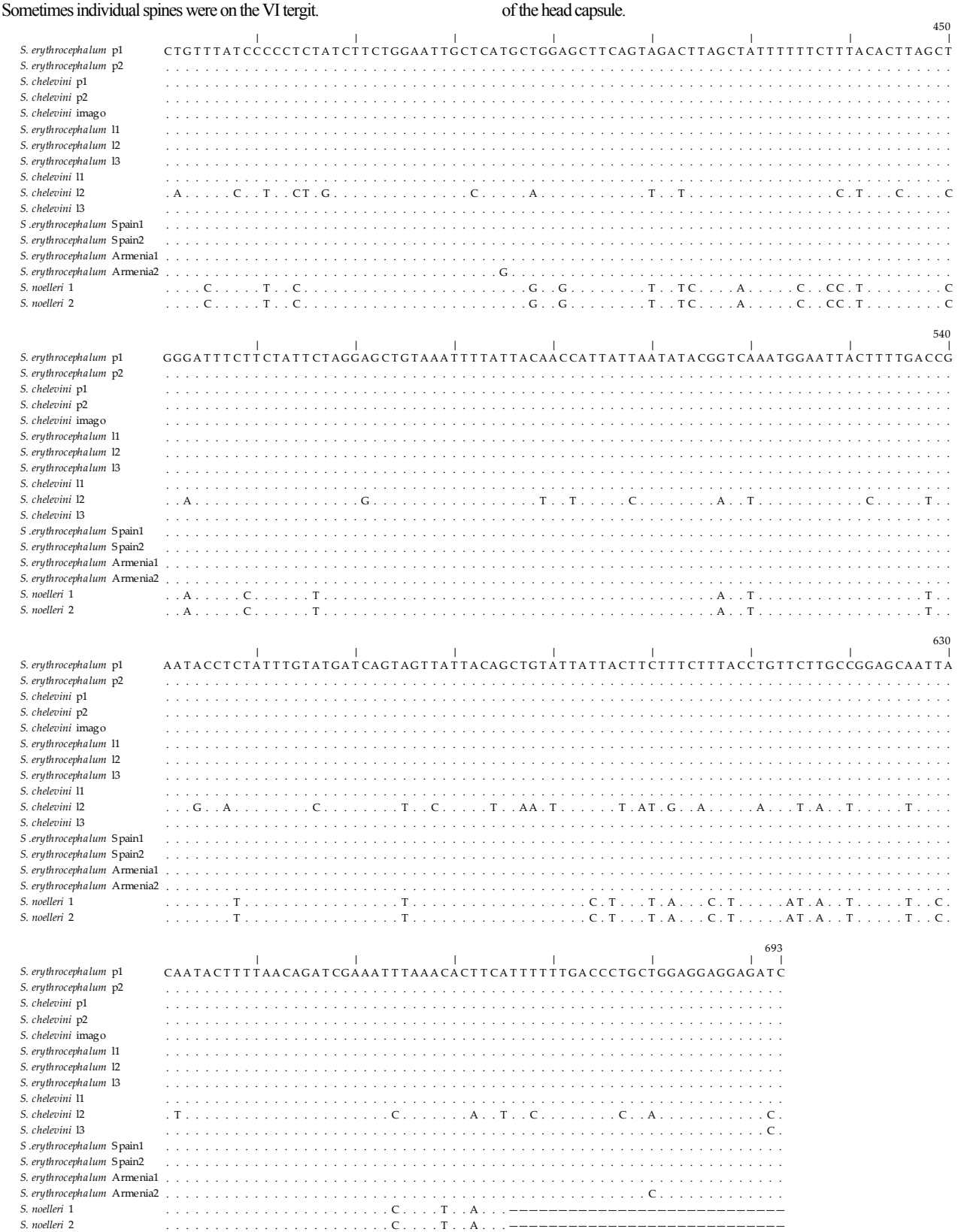

Fig. 10. Alignment of 615 bp COI gene for 17 samples, 361–693 nucleotides: a period indicates the site identical to *S. erythrocephalum* (Ukraine); a dash indicates a gap site; *p* – pupa, *l* – larvae, *1*, *2*, *3* – numbers of samples

In the monograph of Rubtsov (1956) the assumption is made that the large spring and small summer-autumn forms of *S. erythrocephalum* are representatives of different species. The large spring form is a typical representative of *S. sericatum* (Meigen, 1818), and the small summer form is a typical form of *S. erythrocephalum*. Our studies (Sukhomlin & Zinchenko, 2007; Kaplich et al., 2015) did not find significant differences

in the morphology of these two forms, except for the difference in size, which may be related to the peculiarities of nutrition and timing of immature stages.

The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model (Nei & Kumar). The tree with the highest log likelihood (–913.28) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 7 nucleotide sequences. There were a total of 657 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Tamura et al., 2007; Kumar et al., 2018). *Simulium noelleri* Friederichs, 1920 DNA sequences were used as the outgroup.

We determined the samples of the COI gene 615-bp fragments the sequences of twelve samples from different localities and compared them with four samples of *S. erythrocephalum* from Spain and Armenia (Fig. 9, 10). All of the *S. erythrocephalum* samples from Ukraine had 692 bases, the *S. erythrocephalum* samples from Armenia had 673 bases, the *S. noelleri* samples from Ukraine had 664 bases. *S. erythrocephalum* and *S. chelevini* did not have any intraspecific variations, but *S. chelevini* l_2. This may be not a pure sample. These intraspecific variations were not larger than the interspecific variations.

The comparison of two close species of the subgenus *Boophthora* with each other from Volyn (Ukraine) and data from the GenBank (Werner & Kampen, 2012; Andrianov et al., 2015; Ruiz-Arrondo et al., 2018) from Spain and Armenia of the mitochondrial cytochrome c oxidase subunit I (COI) gene are presented in Figure 11.

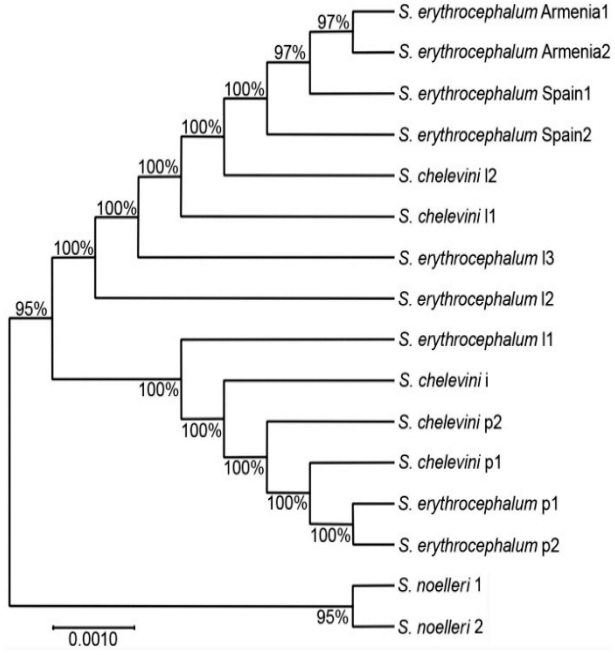

Fig. 11. Phylogenetic relationships of COI haplotypes (Maximum Likelihood method and General Time Reversible model): bootstrap values (>50%) are shown above the branches of clades; *S. erythrocephalum* Spain (GenBank MG894328.1, MG894329.1) (Ruiz-Arrondo et al., 2018), *S. erythrocephalum* Armenia (GenBank KF640027.1, KF640028.1) (Werner & Kampen, 2012; Andrianov et al., 2015); *p* – pupa, *l* – larvae, *1*, *2*, *3* – numbers of samples

On the phylogenetic tree, the data are not grouped, there is no clear separation of the clades. Bootstrap values are 95–100%, which may indicate a significant similarity of all studied samples and the lack of isolation of individual morphotypes from Volyn, Spain and Armenia. Thus, DNA barcoding confirmed the idea that *S. erythrocephalum* and *S. chelevini* are one species, and morphological differences can be considered as manifestations of phenotypic variability.

Conclusions

DNA barcoding did not reveal differences between the two morphologically different species, thus demonstrating its utility to discriminate among morphologically recognized black fly species. In fact, populations of *S. erythrocephalum* and *S. chelevini* from the medium and small rivers of Volyn, Spain and Armenia were found not to be distinct among themselves. Nonetheless, additional molecular techniques together with COI and perhaps other markers may be necessary to overcome difficulties associated with discriminating recently diverged sibling species.

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References

Adler, P. H. (2021). World blackflies (Diptera: Simuliidae): A comprehensive revision of the taxonomic and geographical inventory. Department of Plant and Environmental Sciences, Clemson University, Clemson.

Andrianov, B. V., Goryacheva, I. I., Vlasov, S. V., Gorelova, T. V., Harutyunova, M. V., Harutyunova, K. V., Mayilyan, K. R., & Zakharov, I. A. (2015). Identification of potentially invasive species of black flies (Diptera: Simuliidae) from Armenia based on an analysis of variability in the mt DNA barcode of the cox1 gene and chromosomal polymorphism. Russian Journal of Genetics, 51(3), 289–299.

Bardin, O. (2001). Nuisance due to *Simulium* (*Boophthora*) *erythrocephalum* (De Geer, 1776) (Diptera, Simuliidae) in France. Parasite, 8, 161–162.

Bernotiene, R., & Bartkeviciene, G. (2012). The relationship between water temperature and the development cycle beginning and duration in three black fly species. Journal of Insect Science, 13(1), 1–15.

Chubareva, L. A., & Petrova, N. A. (2008). Citologicheskie karty politennyh hromosom i nekotorye morfologicheskie osobennosti krovososushchih moshek Rossii i sopredel'nyh stran (Diptera: Simuliidae): Atlas [Cytological maps of polytene chromosomes and some morphological peculiarities in the blood-sucking black-flies of Russia and adjacent countries (Diptera: Simuliidae): An atlas]. Research Fellowship KMK, Saint Petersburg (in Russian).

Crease, T. J. (1999). The complete sequence for the mitochondrial genome of *Daphnia pulex* (Cladocera: Crustacea). Gene, 233, 89–99.

Crosskey, R. W. (1990). The natural history of blackflies. Wiley, Chichester.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3(5), 294–299.

Ham, P. J., & Blanco, A. E. (1984). Maintenance of *Simulium* (*Wilhelmia*) *lineatum* Meigen and *Simulium erythrocephalum* de Geer through successive generations in the laboratory. Canadian Journal of Zoology, 62, 870–877.

Ignjatovic-Cupina, A., Zgomba, M., Vujanovic, L., Konjevic, A., Marinkovic, D., & Petric, D. (2006). An outbreak of *Simulium erythrocephalum* (De Geer, 1776) in the region of Novi Sad (Serbia) in 2006. Acta Entomologica Serbica, suppl. 1, 97–114.

Ivashchenko, L. A. (1968). Novye vidy moshek (Simuliidae) [New species of black flies (Simuliidae)]. Parasitology, 12(4), 306–312 (in Rassian).

Jedlička, L., & Halgoš, J. (1982). Daily biting rate of black flies on horses in the Danubian lowlands (Diptera, Simuliidae). Wiodomości Parazytologiczne, 28, 41–44.

Jedlička, L., Kudela, M., & Stloukalova, V. (2004). Key to the identification of blackfly pupae (Diptera: Simuliidae) of Central Europe. Biologia, 59(15), 157–178.

Kaplich, V. M., Sukhomlin, E. B., & Zinchenko, A. P. (2015). Moshki (Diptera: Simuliidae) smeshannyh lesov Evropy [Black flies (Diptera: Simuliidae) of European mixed forests]. Novoe Znanie, Minsk (in Rassian).

Knoz, J. (1965). To identification of Czechoslovakian black-flies (Diptera, Simuliidae). Folia Facultatis Scientiarum Naturalium Universitatis Purkynianae Brunensis. Biologia, 6, 1–52.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 35, 1547–1549.

Nei, M., & Kumar, S. (2000). Molecular evolution and phylogenetics. Oxford University Press, New York.

Niesiołowski, S., & Bokłak, E. (2001). Meszki (Simuliidae, Diptera): Fauna słodkowodna Polski [Black flies (Simuliidae, Diptera): Freshwater fauna of Poland]. Wydawnictwo Uniwersytetu Łódzkiego, Lodź (in Polish).

Panchenko, A. A. (2004). K analizu sistematicheskih i sinonimicheskih nazvanij vidov moshek (Diptera, Simuliidae) Ukrainy [To the analysis of systematic and synonymous names of Ukraine black flies species (Diptera, Simuliidae)]. Visnik Doneckogo Nacionalnogo Universitetu, Seriya A, Prurodnuchi Nauky, 1(2), 404–410 (in Rassian).

Post, R. J. (1983). The annual cycle of *Simulium erythrocephalum* (Diptera: Simuliidae) at a site in Norfolk. Freshwater Biology, 13, 379–388.

Reidelbach, J., & Christl, H. A. (2002). Quantitative investigation into the temporal and spatial variations in the emergence of adult blackflies (Diptera: Simuliidae) from the Breitenbach, a small upland stream in Germany. Limnologica, 32(3), 206–235.

Rubtsov, I. A. (1940). Moshki (sem. Simuliidae). Fauna SSSR. Dvukrylye [Black flies (family Simuliidae). Fauna of the USSR. Diptera]. Academy of Sciences of the USSR, Moscow, Leningrad (in Rassian).

Rubtsov, I. A. (1956). Moshki (sem. Simuliidae). Fauna SSSR. Dvukrylye [Black-flies (family Simuliidae). Fauna of the USSR. Diptera]. Academy of Sciences of the USSR, Moscow, Leningrad (in Rassian).

Ruiz-Arrondo, I., Garza-Hernández, J. A., Reyes-Villanueva, F., Lucientes-Curdi, J., & Rodríguez-Pérez, M. A. (2017). Human-landing rate, gonotrophic cycle length, survivorship, and public health importance of Simulium erythrocephalum in Zaragoza, Northeastern Spain. Parasites and Vectors, 10, 175–183.

Ruiz-Arrondo, I., Hernandez-Triana, L. M., Ignjatovic-Cupina, A., Nikolova, N., Garza-Hernandez, J. A., Rodriguez-Perez, M. A., Oteo, J. A., Fooks, A. R., & Lucientes Curdi, J. (2018). DNA barcoding of blackflies (Diptera: Simuliidae) as a tool for species identification and detection of hidden diversity in the eastern regions of Spain. Parasites and Vectors, 11(1), 463–469.

Sebastiani, F., Meiswinkel, R., Gomulski, L. M., Guglielmino, C. R., Mellor, P. S., Malacrida, A. R., & Gasperi, G. (2001). Molecular differentiation of the Old World *Culicoides imicola* species complex (Diptera, Ceratopogonidae), inferred using random amplified polymorphic DNA markers. Molecular Ecology, 10, 1773–1786.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved PCR primers. Annals of the Entomological Society of America, 87, 1–51.

Sitarz, M., Buczek, A., & Buczek, W. (2021). Skin lesions and systemic reactions in humans infested by blackflies (Diptera: Simullidae) in recreational areas in Southeastern Poland. Journal of Clinical Medicine, 10, 788–796.

Sukhomlin, E., Zinovieva, O., & Kaplich, V. (2019). O patogennyh vidah moshek (Diptera, Simuliidae) podzony smeshannyh lesov Vostochnoj Evropy [About pathogenic species of black flies (Diptera, Simuliidae) of the Eastern Europe mixed forest subzone]. Lesya Ukrainka Eastern European National University Scientific Bulletin, Series Biological Sciences, 387, 81–90 (in Rassian).

Sukhomlin, K. B., & Zinchenko, O. P. (2007). Moshky (Diptera: Simuliidae) Volynskoho Polissia [Black flies (Diptera: Simuliidae) of Volin Polissya]. Vezha, Lutsk (in Ukranian).

Szalanski, A. I., Owens, C. B., Lewter, J. A., & Broce, A. B. (2006). Genetic structure of *Aedes vexans* (Diptera: Culicidae) populations from Central United States based on mitochondrial ND5 sequences. Annals of the Entomological Society of America, 99(1), 157–163.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) soft – ware version 4.0. Molecular Biology and Evolution, 24(8), 1596–1599.

Vujanovic, L., Jovanovic, M., Golusin, Z., Gajinov, Z., & Jakovljevic, S. (2020). Cutaneous manifestations caused by *Simulium erythrocephalum* bites in humans – a case series. Medicinski Pregled, 73, 59–62.

Werner, D., & Kampen, H. (2012). *Simulium* (*Boophthora*) *erythrocephalum* (De Geer, 1776), subgenus and species new to Armenia. Zoology in the Middle East, 56, 85–90.

Yankovsky, A. V. (2002). Opredelitel moshek (Diptera, Simuliidae) Rossii i sopredelnyh territorij (byvshego SSSR) [Keys to black flies (Diptera, Simuliidae) of Russia and adjacent territories (the former USSR)]. Zoological Institute of the Russian Academy of Sciences, Saint Petersburg (in Rassian).

Zinchenko, M. O., Sukhomlin, K. B., Zinchenko, O. P., & Tepliuk, V. S. (2021). The biology of *Simulium noelleri* and *Simulium dolini*: Morphological, ecological and molecular data. Biosystems Diversity. 29(2), 180–184.

Živković, V., & Burány, B. (1972). An outbreak of *Boophthora erythrocephala* (Diptera, Simuliidae) in Yugoslavia in 1970. Acta Veterinaria, 22, 133–142.