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

Sukhomlin, K. B., Zinchenko, M. O., Zinchenko, O. P., Tepliuk, V. S., Biletskyi, Y. V., Biletska, M. G., Buslenko, L. V., Ivantsiv, V. V., & Budnik, S. V. (2022). The biology of *Simulium erythrocephalum* and *S. chelevini* (Diptera, Simuliidae): Morphological, ecological and molecular data. *Biosystems Diversity*, 30(1), 3–11. doi:10.15421/012201

The subgenus *Boophthora* is a typical Palearctic taxon, which includes only 6 species, among them. *Simulium erythrocephalum* has a transpalaearctic 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 (Sty, Chornoguzka, Putylivka) from 2017 to 2019. We used the EPPD 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 Simulidae 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. biulavii* Rubtsov, 1940 (Siberia), *S. erythrocephalum* (De Geer, 1776) (Transpalaearctic), *S. giyuyangense* Chen, Liu, Yang, 2016 and *S. quattuorfile* Chen, Wu, Yang, 2010 (China), *S. ma- kunbei* (Oono, 1977), 2010 and *S. yowgoense* 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* Ivanshchenko, 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, Tereziv, Tetevev) and small (Konopelka, Gapa, Vyzhivka) rivers, and small (Konopelka, Gapa, Vyzhivka) streams and meandering 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 apotome; 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-Arrodillo 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-Arrodillo 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 (Knöz, 1965; Živković & Burány, 1972; Jedlička et al., 2004), Northern (Crosskey, 1990; Yankovsky, 2002; Raastad, J. E., Usova, Z. V., & Kruse, K. (2010). Blackflies of Northern Europe (Diptera: Simuliidae), CD-ROM, ETI Bioinformatics: Amsterdam), Eastern (Niesiołowski & Boldak, 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 (Bernoittene & Bartkevičiene, 2012), gonotrophic cycle of the species (Harm & Blanco, 1984; Ruiz-Armondo et al., 2017), cytological map of polytene chromosomes (Chubareva & Petrova, 2008).

Particular attention was paid to the medical and veterinary value of *S. erythrocephalum* (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) Holmbr., 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; 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.
During initial processing of insect samples protocols as recommend-
ded 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 manufac-
turer instructions for Animal Tissue. PCR-settings to amplify the mitochon-
drial 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 Master-
mix using 20 mg/mL BSA was prepared according to the EPPO Stan-
dard. 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
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 frag-
ments were sequenced in both directions by the Sanger method using the
ExoStar kit for sequencing reactions, following the manufacturer’s proto-
col. Sequence configuration was performed by comparing free DNA
strands. Editing of DNA sequences, assembly configuration and align-
ment of consensus sequences and phylogenetic analysis were performed
using MEGA version X (Kumar et al., 2018).

Standard statistical software packages for the personal computer Sta-
tistic10 (StatSoft 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, wide-
spread 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
substrate at 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/dm²) 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.

The clypeus is oval-elongated (length 0.15 mm, width 0.04 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 ±

The maxillary palpus is brown, the 3rd pal-
pomere is longer (0.49 mm), pedicel and flagellum 1 (0.05 mm) 1.5 times
longer than the flagellum 2. The maxillary palpomere is brown, the 3rd pal-
pomere 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. Calcarpa is small (0.015 mm), its width (0.02 mm)
is slightly less than half the width of the basitarsus at the distal end. Pedi-
susculus occupying ⅓ the width of the tarsomere 1. The claw is simple
(length 0.04 mm).

Abdomen. Genital fork with a very long stern (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 outbreak and a notch in the middle of the posterior edge.
Hygopriphal valves have smooth, darkened edges, slightly diverging be-
hind, 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 longer than the flagellum 2
(0.03 mm). The maxillary palpomere 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
delongated along the sides. The anepisternal membrane is not pubescent.
Legs are dark brown. Only the tarsal hind legs in the central part are light.

Biosyst. Divers., 2022, 30(1)
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 $\frac{1}{3}$ the width of the tarsomere 1.

**Abdomen.** Ventral plate is small, lamellate (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. Gonostylius 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 paranumere 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, milkily-yellow, dirty yellow colour, light head (Fig. 5).

**Abdomen.** 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 gills is ½ the width of the basitarsus at the distal end. Pedisulcus occupying $\frac{1}{3}$ the width of the tarsomere 1. The claw is simple.

**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 $\frac{1}{4}$ 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 $\frac{1}{3}$ the width of the basitarsus at the distal end. Pedisulcus occupying $\frac{1}{3}$ 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 hypopygial 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).

**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 cornia and setae on the upper edge.
to the width and occupies the ½ busitarsus at the distal end. Pedisalus 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 parameral subtriangular plate.

Comparison of _S. chelevini_ and _S. erythrocephalum_ 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 palp | 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 palp | (0.47 mm), 5th palpomere (0.20 mm) is equal to the length of the previous two | (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 the 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 tertiaries of the abdomen | less than 4-5 | above 5 |

Our nucleotide sequence of _S. chelevini_ p1 (615 bp)

**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 palp | 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 palp | (0.47 mm), 5th palpomere (0.20 mm) is equal to the length of the previous two | (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 the 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 tertiaries of the abdomen | less than 4-5 | above 5 |

Our nucleotide sequence of _S. chelevini_ p1 (615 bp)

**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 palp | 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 palp | (0.47 mm), 5th palpomere (0.20 mm) is equal to the length of the previous two | (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 the 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 tertiaries of the abdomen | less than 4-5 | above 5 |
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 (Sakhornil & 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 larve overwinter (Karplik 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 tergite.
On VII–VIII – full rows of spines of different sizes, on IX – small spines.
Sometimes individual spines were on the VI tergite.  

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

| S. erythrocephalum p1 | CTCCTTATCCCTACTCTCTGGATTTTCAAGCTATGGTACAGTTGCTTTCTTACCTTTAGCTT |
| S. erythrocephalum p2 |                  |
| S. chelevini p1 |                  |
| S. chelevini p2 |                  |
| S. chelevini image |                  |
| S. erythrocephalum 1 |                  |
| S. erythrocephalum 2 |                  |
| S. chelevini 1 |                  |
| S. chelevini 2 | A.C.T.G. C.A.T.T.C.T. C.T.C.C.T. |
| S. chelevini 3 |                  |
| S. chelevini 4 |                  |
| S. chelevini 5 |                  |
| S. chelevini 6 |                  |
| S. chelevini 7 |                  |
| S. chelevini 8 |                  |
| S. chelevini 9 |                  |
| S. chelevini 10 |                  |
| S. chelevini 11 |                  |
| S. chelevini 12 |                  |
| S. chelevini 13 |                  |
| S. chelevini 14 |                  |
| S. chelevini 15 |                  |
| S. chelevini 16 |                  |
| S. chelevini 17 |                  |
| S. chelevini 18 |                  |
| S. chelevini 19 |                  |
| S. chelevini 20 |                  |

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 – larva, 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; Kaplîch et al., 2015) did not find significant differences

Biosyst. Divers., 2022, 30(1)
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., 2019; 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 1. This may be a pure sample. These intraspecific variations were not larger than the interspecific variations.

The comparison of two close species of the subgenus Boophilus with each other from Volyn (Ukraine) and data from the Genbank (Werner & Kampen, 2012; Andrianov et al., 2015; Ruiz-Armondo 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 MG984328.1, MG984329.1) (Ruiz-Armondo et al., 2018); S. erythrocephalum Armenia (GenBank KF640027.1, KF640028.1) (Werner & Kampen, 2012; Andrianov et al., 2015); p – papa, l – larva, 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.

The authors express their sincere gratitude to V. Gusarov, a leading researcher at the University of Oslo, for the opportunity to take part in the Ukrainian-Norwegian project on DNA barcoding of insects; V. O. Korneev, Head of Entomology and Collection Management Department, I. I. Schmalhausen Institute of Zoology, DSc, PhD, Full Professor, Corresponding Member of NAS of Ukraine; M. O. Kaluzhina Ph. D, junior researcher, Department of Entomophages Systematics and Ecological Fundamentals of Biomed, I. I. Schmalhausen Institute of Zoology NAS of Ukraine; G. V. Popov, Ph. D., senior researcher, Department of Entomology and Collection Management, I. I. Schmalhausen Institute NAS of Ukraine; O. B. Marti nov Ph. D., senior researcher Department of Zoology, the National Museum of Natural History, NAS of Ukraine in organizing and training at the Natural History Museum, University of Oslo.

The research is partially funded by project entitled “Training the new generation of entomologists in DNA-based molecular methods – international network (Ento MOL)”, supported by the Eurasia Programme 2017–2019 of the Norwegian Centre for International Cooperation in Education (SIU) (Project CPEA-LT/2016/10140).

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 mtDNA barcode of the cox1 gene and chromosomal polymorphism. Russian Journal of Genetics, 51(3), 289–292.

Bardeau, O. (2001). Naisance due à Simulium (Boophilus) erythrocephalum (De Geer, 1776) (Diptera, Simuliidae) in France: Parasite, 8, 161–162.

Bemontaine, R., & Bankoviccicene, 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.

Chubarova, L. A., & Petrova, N. A. (2008). Citologicheskie karty politennyh hromosom i nekotorye morfologicheskie osobennosti krovososushchih moshek Rossii (Chromosomes and some morphological peculiarities in the blood-sucking black-flies of Russia). Department of Plant and Environmental Sciences, Clemson University, Clemson.

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.

Fohrer, O., Black, M., Hodz, 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 (Wilhelmina) lineatum Meigen and Simulium erythrocephalum de Geer through successive generations in the laboratory. Canadian Journal of Zoology, 62, 870–877.

Ignjatovic-Cupina, A., Zgoni, M., Vujnovic, L., Kojcic, A., Marinovic, D., & Petro, D. (2006). An outbreak of Simulium erythrocephalum (de Geer, 1776) in the region of Novi Sad (Serbia) in 2006. Acta Entomologica Serbia, suppl. 1, 97–114.

Ivashchenko, L. A. (1968). Novye vidy moshek (Simuliidae) [New species of black flies, Simuliidae]. Som i nekotorye morfologicheskie osobennosti krovososushchih moshek Rossii (Some and some morphological peculiarities in the blood-sucking black-flies of Russia). Department of Plant and Environmental Sciences, Clemson University, Clemson.

Jedlička, L., & Halgoš, J. (1982). Daily biting rate of black flies on horses in the Danubian lowlands (Diptera, Simuliidae). Wiodomošci, 294–299.

Kaliuzhna, M. O., Ignjatovic, A., Zgomba, M., Vujanovic, L., Konjevic, A., Marinkovic, D., & Petrova, N. A. (2008). Citologicheskie karty politennyh hromosom i nekotorye morfologicheskie osobennosti krovososushchih moshek Rossii (Chromosomes and some morphological peculiarities in the blood-sucking black-flies of Russia). Department of Plant and Environmental Sciences, Clemson University, Clemson.

Lukashevich, A. V., Kudela, M., & Stloukalova, V. (2004). Key to the identification of black flies (Diptera: Simuliidae) Continuing studies of S. chelevini – pupa, S. chelevini – larvae, S. chelevini 1 (De Geer, 1776) (Diptera, Simuliidae) in France: Parasite, 8, 161–162.

Petric, D. (2006). An outbreak of Simulium (Wilhelmina) lineatum Meigen and Simulium erythrocephalum de Geer through successive generations in the laboratory. Canadian Journal of Zoology, 62, 870–877.

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

Jedlička, L., & Halgoš, J. (1982). Daily biting rate of black flies on horses in the Danubian lowlands (Diptera, Simuliidae). Wiodomošci, 294–299.

Jedlička, L., Kudola, M., & Štoukalová, V. (2004). Key to the identification of black-fly pupae (Diptera: Simuliidae) of Central Europe. Biologia, 59(15), 157–178.
Biosyst. Divers., 2022, 30(1)