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ICOSIELLA NEGLECTA (NEMATODA, ONCHOCERCIDAE) IN UKRAINE: OCCURRENCE, HOSTS, MORPHOLOGICAL AND MOLECULAR CHARACTERISATION

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Icosiella neglecta **(Nematoda, Onchocercidae) in Ukraine: Occurrence, Hosts, Morphological and Molecular Characterisation. Kuzmin, Yu., Dmytriieva, I. & Svitin, R. —** *Icosiella neglecta* (Diesing, 1851) is a filarial nematode infecting anuran amphibians in the western Palaearctic. In the present survey, we found *I. neglecta* in 27 out of 28 localities in eight regions of Ukraine. In those localities, 56.5 % of sampled green frogs (*Pelophylax esculentus* complex) were infected. The infection prevalence was similar in the two parental species, *P. ridibundus* (Pallas) and *P. lessonae* (Camerano), and their hybrid form, *P. esculentus* (Linnaeus), while the infection intensity was comparatively higher in *P. ridibundus*, as well as in the samples including both *P. ridibundus* and *P. esculentus*. Morphological examination of 40 females and 80 males of *I. neglecta* confirmed the consistency of main qualitative characters, whereas most metrical characters appeared to be rather variable and related to body length. Muscular oesophagus dimensions and spicule length in males were found to be the least variable. In the studied sample of males, 54 % had a longer spicule on the left side and a shorter one on the right side; the other 46 % had a longer spicule on the right side. Our molecular study using mitochondrial COI gene sequences confirmed the strong genetic similarity of *I. neglecta* populations in Ukraine and most other parts of its distribution area. A single nuclear 18S sequence generated was identical to one previously deposited in GenBank, originating from Ukraine.
Key words: *Icosiella*, *Pelophylax*, filaria, morphometry, COI, Ukraine.

Introduction

Icosiella neglecta (Diesing, 1851) is the type-species of the genus *Icosiella* Seurat, 1917. Nine known species of the genus were reported from various parts of Eurasia and northern Africa (Bursey et al., 2003). *Icosiella neglecta* is the only species widely distributed in the western Palaearctic (Bain & Purnomo, 1984; Mikulíček et al., 2021). The species was initially named and briefly described as *Filaria neglecta* Diesing, 1851 from the frog, *Pelophylax esculentus* (Linnaeus), by Diesing (1851). Seurat (1917) erected a separate genus *Icosiella* Seurat, 1917 for this species and gave its diagnosis and description based on the specimens collected from *P. ridibundus* (Pallas) in France. Dinnik (1930) described the species from the same host in Caucasia. Desportes (1941, 1942) investigated the life-cycle of *I. neglecta* in France and gave a brief description of the species. Presently, *I. neglecta* is known as a parasite of Ranidae, mostly *Pelophylax* spp., rarely occurring in *Rana* spp., Discoglissidae, Hylidae, and Bufonidae (Baker, 1987). Mikulíček et al. (2021) investigated the population-genetic structure of *I. neglecta* in its distribution area, from the Iberian Peninsula to Central Asia, and reported low inter-population differentiation. The sample of nematodes studied by these authors included several specimens from Zakarpatska Oblast (administrative region) in western Ukraine.

In Ukraine, *I. neglecta* was first reported by Mazurmovich (1951) from *Pelophylax* spp. near Kyiv, with the infection prevalence being as low as 2.2 %. Later on, the author recorded this species in the same hosts in western Ukraine (Mazurmovich, 1959, 1965). Shevchenko (1966) and Rezvantseva et al. (2010) recorded *I. neglecta* in Kharkiv Oblast in eastern Ukraine. Maguza (1972) found two specimens of *I. neglecta* in one out of 93 studied *P. esculentus* in northern Ukraine (Polissia region). In the same region, Kuzmin et al. (2020) reported the overall infection prevalence of *I. neglecta* as 52.6 % in *P. esculentus* and 40.7 % in *P. ridibundus*.

During the investigation of helminth parasites of amphibians in Ukraine, we collected *I. neglecta* from various hosts in various localities. The available material allowed us (1) to study the occurrence and abundance of *I. neglecta* in different hosts, localities and regions of the country; (2) to compose a detailed description of the species based on representative samples of adult males and females, and to analyse the variability of morphological characters; and (3) to compare molecular data (mitochondrial COI gene and nuclear 18S sequences) of selected *I. neglecta* specimens with those from previous studies. The results of these studies are presented herein.

Material and methods

Hosts (*Pelophylax* spp.) were collected manually during the warm season, from May until October, in 2017–2021. In total, 28 localities were investigated in eight oblasts of Ukraine (fig. 1); each sample contained from eight to 35 frogs (table 1). The entire sample of 421 individuals included 14 *P. lessonae* (Camerano), 96 *P. esculentus* (hybrid form), and 311 *P. ridibundus*. Frogs (mostly adult individuals) were identified based on morphological characters: colouration pattern, resonator colour, shape and size of the metatarsal tubercle, and shin length (Nekrasova, 2002; Nekrasova et al., 2005; Pisanets, 2007). The snout-vent length (SVL) of each frog was measured; in the text, SVL for separate frog samples is presented as mean value ± standard deviation (SD). Collection of amphibians in protected natural areas was licensed by the "Agreement on scientific cooperation between the I. I. Schmalhausen Institute of Zoology and National Natural Park "Prypiat-Stokhid" (2010)", and the "Agreement on scientific cooperation between the I. I. Schmalhausen Institute of Zoology and Rivnenskyi Nature Reserve (2014)". Handling of animals was in compliance with institutional and national guidelines and regulations.

Collected frogs were transported to the laboratory alive, euthanized, and examined for parasites. Their internal organs were removed and studied under a dissection microscope; in addition, the body cavity, body and limb musculature, as well as spaces under the skin were checked for parasites. Specimens of *I. neglecta* were gathered from the hosts' muscles using fine forceps, then rinsed in saline, killed by gentle heating, and fixed in 75 % ethanol. In total, more than 1300 specimens of *I. neglecta* were collected; 120 of them (80 males and 40 females) were used for morphological studies.

The occurrence of *I. neglecta* in the hosts was estimated as the prevalence of infection, i. e. the percentage of infected host individuals in a sample. We analysed several types of samples: (1) samples collected from particular localities listed in table 1; (2) samples collected from separate host species (the hybrid form *P. esculentus* was considered a species); (3) samples including individuals either of one host species or mixed samples including both hybrids and individuals of one of the parental species. For each sample, the 95 % confidence intervals of infection prevalence and mean abundance were calculated in Quantitative Parasitology 3.0 software (Rózsa et al., 2000). The same program was used to estimate differences in infection prevalence between the samples using the unconditional exact test (UET).

Table 1. Survey localities and hosts; prevalence and mean abundance (both shown with 95 % confidence intervals in parentheses) of *Icosiella neglecta* infection in each locality

No.	Locality description and coordinates	Collection month, year	Host species	n	Infection prevalence, %	Mean abundance
	Volyn Oblast					
1.	Small pond in Svityaz' village; 51°28'23.09" N, 23°50'40.29" E	May, 2021	*P. esculentus*	24	66.7 (45.8–83.1)	2.29 (1.46–3.46)
2.	Pisochne Lake; 51°34'4.96" N, 23°55'27.40" E	September, 2018	*P. ridibundus* + *P. esculentus*	13	46.2 (22.4–73.9)	2.38 (0.92–4.46)
3.	The Pripyat River near Vetly village; 51°52'41.99" N, 25° 6'57.27" E	August, 2019	*P. ridibundus* + *P. esculentus*	10	40 (15.0–70.9)	2.3 (0.6–6.7)
4.	Small artificial lake close to Liubiaz' Lake; 51°48'45.70" N, 25°28'52.12" E	June, 2017	*P. esculentus*	10	80 (44.6–96.3)	2.3 (1.2–3.5)
	Rivne Oblast					
5.	Reclamation canals in Perebrody area of Rivnenskyi Nature Reserve; 51°39'2.97" N, 27°4'55.00" E	May, 2018	*P. lessonae* + *P. esculentus*	8	12.5 (0.6–50)	1.38 (0–2.75)
6.	Forest ponds in Somyne area of Rivnenskyi Nature Reserve; 51°24'9.57" N, 26°51'4.86" E	May, 2018	*P. lessonae* + *P. esculentus*	11	81.8 (50.0–96.7)	1.91 (1.0–2.73)
7.	Reclamation canals in the forest near Smyha village; 50°14'15.57" N, 25°47'54.20" E	May, 2020	*P. ridibundus* + *P. esculentus*	11	100 (73.5–100)	12.64 (8.82–17.36)
	Zhytomyr Oblast					
8.	Reclamation canals near Khodaky and Berestovets villages; 50°59'10.72" N, 28°51'20.00" E	May, 2018	*P. lessonae* + *P. esculentus*	10	30 (8.7–61.9)	0.3 (0–0.5)
9.	Dam on the Uzh River near the town of Korosten; 50°59'25.31" N, 28°41'45.66" E	May, 2018; June, 2019	*P. ridibundus*	20	90 (72.5–97.3)	4.1 (2.8–6.3)
10.	Dam on the Irsha River in the town of Malyn; 50°46'12.69" N, 29°12'10.18" E	July, 2019	*P. ridibundus*	10	90 (55.3–99.5)	13.3 (6.2–23.7)
11.	The Teteriv River near the town of Korostyshiv; 50°18'14.86" N, 29°4'22.54" E	August, 2020	*P. ridibundus*	15	53.3 (29.4–77.8)	4.4 (1.47–12.0)
	Kyiv Oblast					
12.	Fishery pond, western edge of Kyiv; 50°27'36.90" N, 30°17'37.69" E	September, 2016; August, 2018	*P. ridibundus* + *P. esculentus*	22	22.7 (9.4–45.3)	0.41 (0.14–0.95)
13.	Forest lake near Lisnyky village; 50°18'21.25" N, 30°32'24.60" E	May, 2017	*P. lessonae*	8	37.5 (11.1–71.1)	0.63 (0.13–1.5)

14. Ponds in Holosiyivskyi Park, Kyiv; 50°23'12.57" N, 30°29'49.80" E	September, 2018	*P. ridibundus*	10	90 (60.2–98.9)	8.8 (4.5–14.1)
15. Pond near the village of Vasyliv; 49°57'18.55" N, 30°27'24.83" E	October, 2018	*P. ridibundus*	15	33.3 (14.2–60.3)	0.6 (0.13–1.27)
16. Pond in Bilihorodka village; 50°22'30.89" N, 30°13'47.93" E	June, 2019	*P. ridibundus*	15	86.7 (60.3–97.6)	5.0 (3.13–7.6)
17. Inlet of the Dnipro River near Trypillia village; 50°7'16.43" N, 30°46'49.00" E	June, 2019	*P. ridibundus*	6	83.3 (41.1–99.1)	2.17 (0.67–3.0)
18. Tel'bin Lake, Kyiv; 50°25'30.87" N, 30°36'37.04" E	September, 2018; September, 2019	*P. ridibundus*	24	87.5 (69–96.5)	6.25 (4.38–8.58)
19. Tyahle Lake, southern edge of Kyiv; 50°22'46.93" N, 30°38'23.90" E	June, August, 2020	*P. ridibundus*	33	84.8 (68.4–93.8)	6.21 (4.45–8.42)
20. Inlet of the Dnipro River, southern edge of Kyiv; 50°22'7.31" N, 30°34'20.39" E	July, 2021	*P. ridibundus*	20	20 (7.1–42.3)	0.9 (0.1–3.15)
21. Bank of the Dnipro River near the town of Rzhyshchiv; 49°58'26.08" N, 31° 3'53.53" E	August, 2021	*P. ridibundus*	21	66.7 (44.9–84.1)	4.43 (2.62–7.95)
Poltava Oblast					
22. Swamp in Pyryatynskyi National Nature Park; 50°16'55.43" N, 32°31'36.54" E	July, 2019	*P. ridibundus* + *P. esculentus*	12	33.3 (12.3–63.0)	1.0 (0.17–3.17)
23. The Vorskla River in Poltava City; 49°34'15.00"N, 34°35'26.38" E	August, 2021	*P. ridibundus*	10	50 (22.2–77.7)	3.9 (1.4–7.9)
Odesa Oblast					
24. The Dnister River near Mayaky village; 46°25'24.49" N, 30°14'51.39" E	September, 2020	*P. ridibundus* + *P. esculentus*	17	29.4 (12.4–54.4)	1.06 (0.29–2.18)
Mykolaiv Oblast					
25. The Pivdenniy Buh River near Pisky village; 47°8'52.24" N, 31°50'58.34" E	August, 2018	*P. ridibundus*	10	10 (0.5–44.6)	0.1 (0–0.3)
26. Pond near Bohdanivka village; 47°49'15.06" N, 31°5'54.90" E	August, 2019	*P. ridibundus*	13	7.7 (0.4–34.1)	0.15 (0–0.46)
27. The Pivdenniy Buh River near Myhiya village; 48°1'47.17" N, 30°56'58.46" E	July, 2020	*P. ridibundus*	15	40 (19.1–66.8)	0.67 (0.27–1.2)
Kherson Oblast					
28. Ponds at irrigation canal in Hryhorivka village; 46°16'20.58" N, 33°43'24.25" E	August, 2021	*P. ridibundus*	28	0	0

Similarly, the abundance of *I. neglecta* in separate samples was characterised by the infection intensity, i. e. the number of nematodes in infected hosts, and the mean abundance, i. e. mean number of nematodes in all hosts in a sample including uninfected hosts. Mean and median intensity, as well as minimum and maximum values were used for the infection intensity presentation in separate samples. To compare infection intensity between samples, we used the Mann-Whitney U test for medians; calculations were performed in PAST 3.1 software (Hammer et al., 2001). The same program was used for calculation of correlation between host size (SVL) and *I. neglecta* infection intensity using Spearman's correlation coefficient (r_S).

For morphological studies, nematodes stored in 75 % ethanol were placed in distilled water for 15–30 minutes, and then cleared in lactophenol (mixture of equal volumes of water, glycerine, phenol, and lactic acid) for 1–4 hours. Specimens of *I. neglecta* were studied as temporary mounts in lactophenol under a Zeiss Axio Imager M1 microscope equipped with a digital imaging system and differential interference contrast (DIC). Measurements and photomicrographs were taken with the aid of digital imaging software Zeiss AxioVision40 version 4.6.3; line drawings were made based on digital images. Apical and transverse sections were cut manually with a thin blade. All measurements in the text are in micrometres unless otherwise stated. Minimum and maximum values of metrical characters are presented in the text of descriptions as ranges; mean value, SD, and coefficient of variation are given in tables 3 and 4 for each character. Correlation between separate characters and body length in males and females was determined using Pearson correlation coefficient (r_P) calculated in PAST 3.1. Mean values of metrical characters in the two types of males differing in the position of spicules were compared using t-test calculated in PAST 3.1.

For scanning electron microscopy (SEM) we used the anterior part of one female specimen. Following Netherlands et al. (2020), the specimen was dehydrated in a series of ethanol (70 %, 80, 90, 96 and 100 %) mixed 1 : 1 with hexamethyldisilazan with final drying in clear hexamethyldisilazan. The dried specimen was mounted onto 12 mm aluminium stubs with double-sided carbon tape and sputter-coated for 90 seconds with a gold palladium alloy in argon gas at a pressure of 2 atm (SPI-ModuleTM Sputter Coater, SPI Supplies, West Chester, PA, USA). Specimen stubs were stored in a desiccator for at least 30 min before being examined by SEM at an accelerated voltage of 10 kV (Phenom PRO Desktop SEM, Phenom-World B., Eindhoven, Netherlands).

The middle fragment of one male specimen collected from *P. ridibundus* in the Tel'bin Lake, Kyiv (table 1) was used for molecular studies. DNA was extracted using the PCRBio Rapid Extraction Kit (PCR Biosystems Ltd, London, Great Britain) following the manufacturer's instructions. Following Lefoulon et al. (2015), Cytochrome oxidase c subunit I (COI) amplicons were obtained using the primer pair COIintF (5´-TGA TTG GTG GTT TTG GTA A-3´) and COIintR (5´-ATA AGT ACG AGT ATC AAT ATC-3´), and the ribosomal small subunit (18S rDNA) ones were obtained using the primer pair F18ScF1 (5´-ACC GCC CTA GTT CTG ACC GTA AA-3´) and F18ScR1 (5´-GGT TCA AGC CAC TGC GAT TAA AGC-3´). The thermocycling profiles were also in accordance with Lefoulon et al. (2015). PCR products from each sample were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) for purification and sequencing in both directions. Resultant sequences were assembled, and chromatogram-based contigs were generated and trimmed using Geneious Prime software (www.geneious.com) and submitted to GenBank. The pairwise and UPGMA analyses were performed using Mega (V. 11) software (Tamura et al., 2021).

Results

1. Localities

Icosiella neglecta was recorded in 27 of 28 studied localities (fig. 1; table 1); it was absent in a sample from one locality (no. 28; n = 28) in Kherson Oblast in southern Ukraine. The latter sample of frogs was excluded from further analysis. In four other samples collected in the southern part of the country (nos. 24–27; Odesa and Mykolaiv Oblasts), the prevalence of infection was less than 50 %, and the mean abundance exceeded 1.0 only in a sample from Odesa Oblast. In the northern part of Ukraine (Volyn, Rivne, Zhytomyr, and Kyiv Oblasts), both infection prevalence and abundance were generally higher, 62.0 % and 3.9 on average. Interestingly, high infection prevalence and abundance were observed in localities situated in natural or less anthropogenically transformed biotopes, e.g. locality no. 7, as well as on urbanised territories in cities, towns, and villages, e. g. localities nos. 10, 16, 18, and 19. The prevalence of infection and abundance were comparatively moderate (33.3–50 %) in two samples collected in Poltava Oblast in eastern Ukraine (table 1).

Fig. 1. Studied localities in Ukraine. Numbers correspond to those in table 1.

2. Hosts

Icosiella neglecta was found in 222 (56.5 %) of 393 examined frogs from 27 localities. The mean infection intensity was 6.0, the median was 4.0. We recorded *I. neglecta* in all three members of the *Pelophylax esculentus* complex: *P. lessonae*, *P. ridibundus*, and the hybrid form *P. esculentus*. Infection prevalence was similar in all three hosts (table 2). Despite the prevalence being somewhat lower in *P. lessonae*, we found no statistical differences between the prevalence in the three hosts (UET $p = 1$). Infection intensity was similar in *P. lessonae* and *P. esculentus* but markedly higher in *P. ridibundus* (table 2). The difference in median infection intensity between *P. ridibundus* and *P. esculentus* was statistically significant (Mann-Whitney test $p < 0.01$). The difference in infection intensity between

Table 2. Infection prevalence (shown with 95 % confidence intervals in parentheses) and intensity (shown as mean, median in brackets and range in parentheses) in separate host species and types of populations of *Pelophylax* spp. in Ukraine

Host species / Population type	n	Frog size (SVL), mm	Infection prevalence, %	Infection intensity
Species:				
P. lessonae	14	62.1 ± 6.5 (53–75)*	50 (23.8–76.2)	3.4 [3] (1–11)
P. esculentus	96	67.2 ± 12.6 (38–97)	56.2 (45.8–66.2)	3.8 [3] (1–22)
P. ridibundus	283	72.4 ± 14.5 (35–111)	56.9 (51.1–62.5)	6.8 [5] (1–42)
Population type:				
P. lessonae	8	59.4 ± 4.8 (53–66)	37.5 (11.1–71.1)	1.7 [1] (1–3)
P. esculentus	34	73.3 ± 9.3 (52–97)	70.6 (52–84.3)	3.2 [3] (1–10)
P. ridibundus	237	73 ± 14.8 (38–111)	62 (55.7–68.2)	6.7 [4] (1–42)
P. lessonae + P. esculentus	29	60.2 ± 12.2 (38–82)	44.8 (27.2–64)	2.7 [1] (1–11)
P. ridibundus + P. esculentus	84	68.3 ± 12.4 (35–95)	40.5 (30.3–51.2)	6.7 [4.5] (1–27)

*Mean ± SD (minimum–maximum).

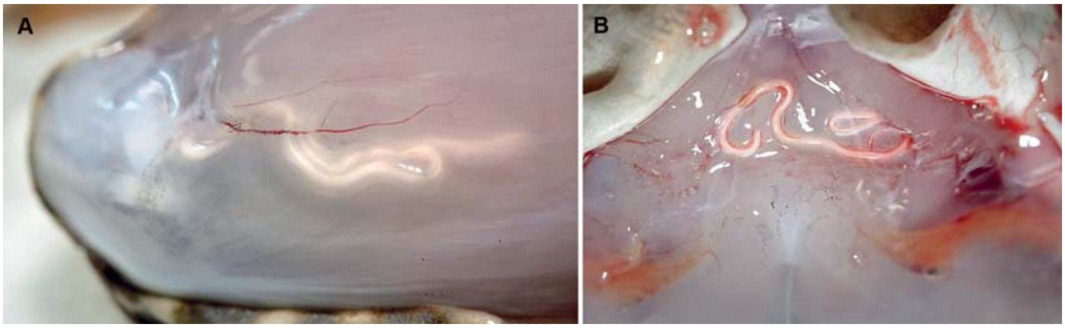

Fig. 2. *Icosiella neglecta* in host leg (A) and throat (B) muscles.

P. lessonae and *P. ridibundus* was not confirmed by statistical tests due to the small *P. lessonae* sample size (n = 14, 7 of them infected).

The examined samples of frogs were composed of the individuals of one species, the hybrids, or both the hybrids and the individuals of either of the parental species (see table 1). Therefore, we distinguished five types of host samples in the examined material corresponding to five types of *Pelophylax* spp. populations (table 2). Infection prevalence and intensity were lowest in a sample of *P. lessonae*. However, the small size of the sample (8 specimens, 3 of them infected) did not allow a reliable statistical comparison of this sample with other types of samples. Infection prevalence in mixed populations (*P. lessonae* + *P. esculentus* and *P. ridibundus* + *P. esculentus*) was statistically similar (UET p = 1) and lower than 50 % (table 2). Samples from the populations of *P. ridibundus* and *P. esculentus* had a higher prevalence of infection. The difference in infection prevalence between the latter two types of samples was not significant (UET p = 0.36). We found a statistically significant difference in the infection prevalence between the samples of *P. esculentus* and *P. lessonae* + *P. esculentus* (UET p = 0.04), *P. esculentus* and *P. ridibundus* + *P. esculentus* (UET p < 0.01), and *P. ridibundus* and *P. ridibundus* + *P. esculentus* (UET p < 0.01). The difference between the samples of *P. ridibundus* and *P. lessonae* + *P. esculentus* was not statistically significant (UET p = 0.08) despite the infection prevalence being higher in the former type of samples (table 2).

Infection intensity was found to be highest in the samples of *P. ridibundus* and the samples consisting of *P. ridibundus* + *P. esculentus* (table 2). These two types of samples had the highest similarity in infection intensity (Mann-Whitney test p = 0.99). The samples of *P. esculentus* and *P. lessonae* + *P. esculentus* had a similar (Mann-Whitney test p = 0.19) and comparatively lower infection intensity. The difference in the *I. neglecta* infection intensity between other pairs of samples was statistically significant (p < 0.05) based on the Mann-Whitney test.

In the entire sample of *Pelophylax* spp., *I. neglecta* was found slightly more often in males (n = 237; SVL = 68.8 ± 11.7 mm; P = 58.6 %) than in females (n = 156; SVL = 73.8 ± 16.8 mm; P = 53.2 %). However, the difference in infection prevalence between male and female frogs was not significant (UET p = 0.29). The infection intensity, on the contrary, was higher in females (mean 6.7, median 5) than in males (mean 5.6, median 4). The difference in median intensities was significant (Mann-Whitney test p = 0.02). Similar results were obtained for the sample of *P. ridibundus*. Males (n = 155; SVL = 69.8 ± 11.6 mm) had a higher infection prevalence (P = 59.4 %) than females (n = 127; SVL = 76 ± 16.5 mm; P = 54.3 %), while infection intensity in males was lower (mean 6.6, median 4) than in females (mean 7.2, median 5). However, the differences in *I. neglecta* infection parameters between male and female *P. ridibundus* were not statistically significant.

In the entire sample of *Pelophylax* spp. (n = 393; SVL = 70.7 ± 14.1 mm), a positive correlation between frog SVL and *I. neglecta* abundance was revealed: $r_S = 0.57$ ($p < 0.01$). In the sample of infected hosts (n = 222; SVL = 76.5 ± 12.5 mm), the correlation between host body size and *I. neglecta* infection intensity was somewhat lower ($r_S = 0.47$) but still significant ($p < 0.01$). A similar correlation was found in the sample of *P. ridibundus* (n = 283; SVL = 72.4 ± 14.5 mm). In the entire sample, the correlation coefficient was 0.59 ($p < 0.01$), while in the sample of infected frogs (n = 161; SVL = 78.7 ± 12.6 mm), the correlation coefficient was 0.4 ($p < 0.01$).

3. Sites of infection

In most frogs, *I. neglecta* was found under the fascias of skeletal muscles. Rarely, in some heavily infected frogs, few *I. neglecta* specimens were observed moving freely in the body cavity and between internal organs. The majority of worms were located in the thigh muscles (fig. 2, A). In about half of the examined frogs, *I. neglecta* specimens were also found in the submaxillary muscles (fig. 2, B), from the lower jaw to the episternum, and under the coracoids and the sternum. Very rarely, the nematodes were located in the abdominal muscles.

4. Morphology

General. Body elongated, thread-like, anterior and posterior ends rounded. Females approximately two times larger than males. Body cuticle thin, with smooth surface. Oral opening circular (fig. 3, B, C). On apical surface, four large submedian papillae; four sclerotised cuticular spikes located in pairs between dorso-lateral and ventro-lateral papillae (figs 3, A, B; 4, A, B). Each spike with wide base, pointed tip, and flat ”root”of irregular shape beneath cuticle. Four minute submedian papillae located just posterior to large ones. Amphids lateral, amphidial openings circular (fig. 3, B, F). Connection between oral opening

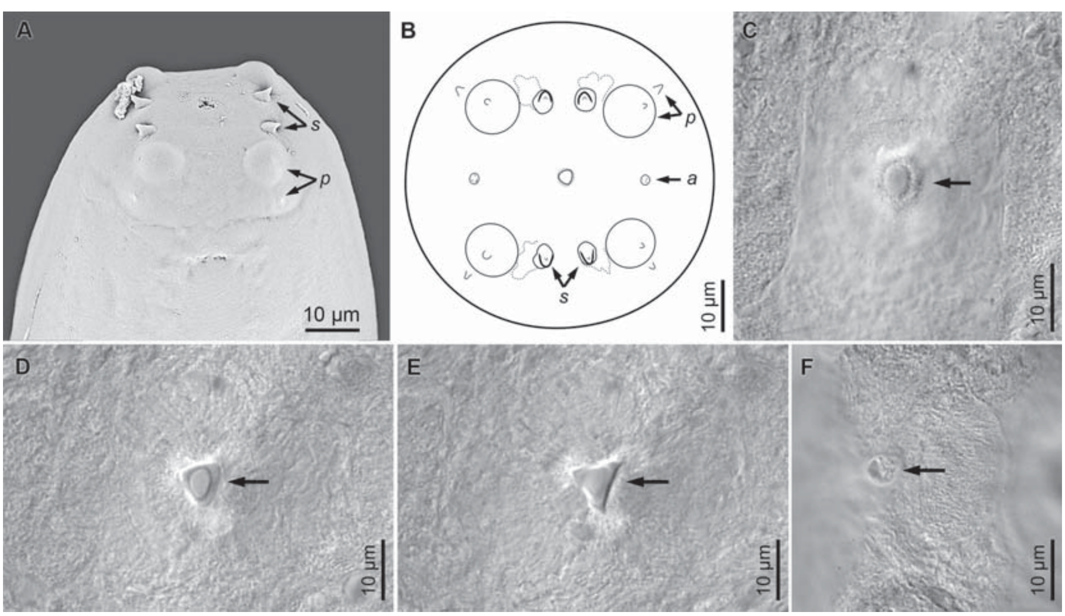

Fig. 3. Apical morphology of *Icosiella neglecta*: A — scanning electron microscopy of apical extremity; B — *en face* view of apical extremity; C — oral opening (arrow); D — cuticularised ring between oral opening and oesophagus (arrow); E — entrance to oesophageal lumen (arrow); F — amphidial opening (arrow); *s* — apical spikes, *p* — papillae, *a* — amphid.

Table 3. Morphometry of *Icosiella neglecta* females (n = 40) from *Pelophylax* spp. in Ukraine

Characters	Mean ± SD	CV, %	r_p
Body length (BL), mm	24.9 ± 7.9	31.6	–
Body width at posterior end of muscular oesophagus	230 ± 54.8	23.8	0.95 (p < 0.01)
Body width at posterior end of glandular oesophagus (BW)	279 ± 75.0	26.9	0.95 (p < 0.01)
Length of muscular oesophagus	416 ± 72.0	17.3	0.77 (p < 0.01)
Length of glandular oesophagus, mm	4.0 ± 1.1	26.6	0.88 (p < 0.01)
Total length of oesophagus (OeL), mm	4.5 ± 1.1	25.3	0.89 (p < 0.01)
Width of muscular oesophagus at anterior end	58 ± 14.2	24.5	0.88 (p < 0.01)
Width of muscular oesophagus at mid-length	67 ± 14.3*	21.4	0.74 (p < 0.01)
Width of muscular oesophagus at isthmus	36 ± 6.7	18.7	0.72 (p < 0.01)
Distance from anterior end to nerve ring	259 ± 51.2	19.7	0.8 (p < 0.01)
Distance from anterior end to vulva	788 ± 222.7	28.3	0.87 (p < 0.01)
Distance from anus to tail tip	57 ± 12.8	22.4	0.67 (p < 0.01)
Ratios:			
Length of muscular oesophagus / OeL, %	9.6 ± 1.5	15.7	–0.58 (p < 0.01)
OeL / BL, %	18.7 ± 4.0*	21.3	–0.7 (p < 0.01)
BW / BL, %	1.1 ± 0.1	11.6	–0.7 (p < 0.01)
Distance to vulva / BL, %	3.3 ± 0.6*	19.4	–0.63 (p < 0.01)

Note. Measurements in micrometres unless otherwise stated; CV — coefficient of variation, r_p — Pearson coefficient of correlation with body length.

*Distribution was not normal according to Shapiro-Wilk test.

and anterior end of oesophagus cuticularised, triangular with rounded corners in apical view (fig. 3, D). Entrance to oesophageal lumen triangular (fig. 3, E). Body wall thickened in apical part (fig. 4, A, B). Muscular oesophagus short, its anterior end rounded (figs 4, A, B; 5, A; 6, B). Lumen of muscular oesophagus tri-radiate (fig. 4, C). At mid-length of muscular oesophagus, three large transparent cells, each located in separate sector of oesophagus. In some specimens, these cells inflated, muscular oesophagus dilated at mid-length. Posterior to mid-length, muscular oesophagus constricted, then gradually widening posteriorly (figs 5, A; 6, B). Constricted part (isthmus) encircled by nerve ring. Glandular oesophagus wide and long, with narrow lumen of irregular shape and numerous large prominent nuclei peripheral in location (fig. 4, D, C). Anterior part of glandular oesophagus surrounding posterior end of muscular oesophagus; on dorsal side, anterior end of glandular oesophagus slightly extended anteriorly (figs 5, A; 6, B). Intestine narrower than glandular oesophagus, intestinal lumen wide. Rectum thin, elongated, poorly visible in both sexes.

Females. [Description based on 40 specimens; measurements in the text are given as ranges; statistical representation of metrical characters is given in table 3]. Body 8.2–38.1 mm long, gradually widening from anterior end till level of posterior end of oesophagus; body width remaining approximately constant till caudal part. Body width 118–331 at posterior end of muscular oesophagus, 128–415 at posterior end of glandular oesophagus. Apical spikes large, conspicuous in lateral view. Muscular oesophagus 268–544 long (6.7–13.0 % of total oesophagus length), 34–85 wide at anterior end, 43–116 wide at mid-length, 24–49 wide at isthmus. Glandular oesophagus 2.29–6.37 mm long; total length of oesophagus 2.6–6.9 mm, or 13.8–31.7 % of body length. Distance from anterior end to nerve-ring 143–364.

Vulva ventral, slightly elevated, with oval aperture, located at level of anterior part of glandular oesophagus (fig. 5, A), 420–1,327 from anterior end; this distance corresponding to 2.4–5.2 % of body length. Vagina short, globular, thick-walled (figs 4, D; 5, D). Wall of vagina vera containing circular muscles; its lumen thin, cuticularised. Lumen of vagina uterina

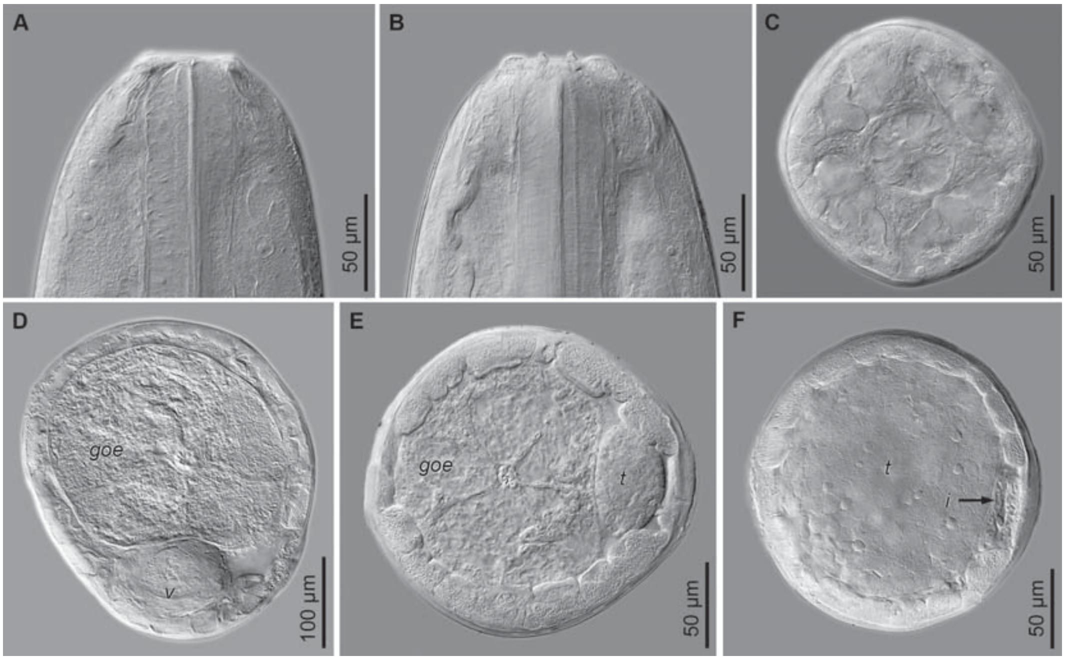

Fig. 4. Anterior end (A, B) and transverse sections (C–F) of *Icosiella neglecta*: A — anterior end in lateral view; B — anterior end in dorso-ventral view; C — transverse section through mid-region of muscular oesophagus; D — transverse section through glandular oesophagus (*goe*) and vagina (*v*) of female; E — transverse section through glandular oesophagus (*goe*) and anterior part of testis (*t*) of male; F — transverse section through mid-body of male showing testis (*t*) and intestine (*i*).

widening posteriorly. Ovejector thick-walled in proximal part (fig. 5, A); wall of ovejector consisting of dense outer layer and inner layer composed by pyramidal cells with prominent nuclei (fig. 5, D). Thickness of ovejector wall decreasing posteriorly. Ovejector splitting into two uteri just posterior to oesophago-intestinal junction. Uteri occupying most space in body posterior to oesophagus, forming numerous loops; in anterior half of body, uteri usually filled with microfilariae. In posterior part, close to caudal region, two elongated, sac-like, thin-walled seminal receptacles filled with numerous spermia (fig. 5, B). Ovaries narrow, their proximal ends drop-shaped (fig. 5, C). Caudal region usually with loops of uteri and ovaries inside, caudal extremity thick-walled, with prominent muscles (fig. 5, B, E). Rectum long, thin-walled; anus sub-terminal, located 30–92 from tail tip (fig. 5, E).

Variability. The topology of the reproductive system was somewhat variable in examined females. In most specimens, the ovejector was directed posteriorly and located along the glandular oesophagus (fig. 5, A). In some specimens, however, the ovejector formed several loops, some of them could reach the level of the posterior part of the muscular oesophagus. Metrical characters in females were rather variable (table 3). Body length had the highest variability (CV = 31.6 %). All direct measurements showed significant positive correlation ($r_p > 0$) with body length, while ratios showed negative correlation ($r_p < 0$) with body length and were less variable compared to direct measurements (table 3). The highest correlation ($r_p = 0.95$) was recorded between body width and body length. The width of the isthmus of the muscular oesophagus was comparatively less variable (CV = 18.7 %) and less correlated with body length ($r_p = 0.72$). The distance from anus to tail tip showed the lowest correlation with body length (r = 0.67), while its variability was still high (CV = 22.4 %). The ratio of body width at the posterior end of the oesophagus to

Fig. 5. Females (A–E) and microfilariae (F) of *Icosiella neglecta*: A — anterior part of body, right lateral view; B — posterior part of body, left lateral view; C — proximal end of ovary (*ov*) in posterior part of body; D — region of vulva, right lateral view; E — posterior end of body, left lateral view; F — microfilariae from ovejector; *moe* — muscular oesophagus, *nr* — nerve-ring, *goe* — glandular oesophagus, *v* — vulva, *oj* — ovejector, *sr* — seminal receptacle, *vv* — vagina vera, *vu* — vagina uterina, *r* — rectum, *a* — anus.

body length showed the lowest variability (CV = 11.6%) and comparatively low correlation with body length (r = -0.7).

Males. [Description based on 80 specimens; measurements in the text are given as ranges; statistical representation of metrical characters is given in table 4]. Body 4.9–16.4 mm long. Posterior part of body curved ventrally (fig. 6, A). Body width 100–258 at posterior end of muscular oesophagus, 110–234 at oesophago-intestinal junction. Apical spikes smaller than in females, inconspicuous in lateral view in some specimens. Muscular oesophagus 238–540 long (7.5–15.2 % of total oesophagus length), 27–60 wide at anterior end, 34–73 wide at mid-length, and 19–39 wide at isthmus. Glandular oesophagus 1.4–4.4 mm long; total length of oesophagus 1.7–4.9 mm, or 23.2–40.1 % of body length. Nerve-ring 155–386 from anterior end.

Testis single, its anterior part narrow (fig. 4, E), reflexed, located posterior to or at level of glandular oesophagus, in some specimens forming several loops and winding around oesophagus. Posterior part of testis wide, occupying almost entire space in mid-region of body (fig. 4, F). Ejaculatory duct narrower than testis (fig. 6, C), *vas deferens* approximately as long as longer spicule. Spicules unequal and dissimilar (fig. 6, C, D). Each spicule enclosed with thin-walled sheath. Longer spicule 253–376 long; shorter spicule 94–142 long, 2.1–3.6 times shorter than longer spicule. Longer spicule with small cup-like head (*capitulum*); shaft (*calomus*) transversely striated, half as long as spicule; blade (*lamina*) thin, slightly curved ventrally, with wide velum not reaching spicule tip. Shorter spicule robust and wide, with flattened

Table 4. Morphometry of *Icosiella neglecta* males from *Pelophylax* spp. in Ukraine, and comparison between two types of males with different position of spicules

Characters	Entire sample (n = 80)			Males with shorter right spicule (n = 43)	Males with shorter left spicule (n = 37)	t-test p
	mean ± SD	CV, %	r_p	mean ± SD	mean ± SD	
Body length (BL), mm	11.2 ± 2.3	20.7	–	11.2 ± 2.1	11.2 ± 2.5	0.91
Body width at posterior end of muscular oesophagus	168 ± 36.3	21.6	0.9 (p < 0.01)	170 ± 35.7	167 ± 37.5	0.69
Body width at posterior end of glandular oesophagus (BW)	182 ± 31.2	17.1	0.87 (p < 0.01)	185 ± 29.3	180 ± 33.4	0.48
Length of muscular oesophagus	361± 65.4	18.1	0.83 (p < 0.01)	366 ± 67.3	356 ± 63.5	0.48
Length of glandular oesophagus, mm	3.2 ± 0.7	22.1	0.86 (p < 0.01)	3.2 ± 0.7	3.2 ± 0.8	0.59
Total length of oesophagus (OeL), mm	3.5 ± 0.8	21.3	0.87 (p < 0.01)	3.6 ± 0.7	3.5 ± 0.8	0.57
Width of muscular oesophagus at anterior end	43 ± 7.5	17.4	0.77 (p < 0.01)	43 ± 6.6	42 ± 8.4	0.41
Width of muscular oesophagus at mid-length	49 ± 7.2	14.6	0.68 (p < 0.01)	50 ± 6.5	49 ± 8.1	0.83
Width of muscular oesophagus at isthmus	28 ± 4.4	15.9	0.74 (p < 0.01)	28 ± 4.6	28 ± 4.4	0.9
Distance from anterior end to nerve ring	236 ± 46.6	19.7	0.85 (p < 0.01)	237 ± 49.1	235 ± 44.2	0.85
Longer spicule length	315 ± 25.9	8.2	0.29 (p < 0.01)	317 ± 23.8	313 ± 28.3	0.54
Shorter spicule length	117 ± 11.6	9.9	0.04 (p = 0.72)	117 ± 12.9	117 ± 10	0.98
Distance from cloacal aperture to tail tip	53 ± 5.8	10.9	0.64 (p < 0.01)	54 ± 5.9	53 ± 5.8	0.53
Ratios:						
Length of muscular oesophagus / OeL, %	10.4 ± 1.4	13.2	-0.38 (p < 0.01)	10.3 ± 1.1	10.5 ± 1.6	0.62
OeL / BL, %	31.7 ± 3.4	10.6	-0.2 (p = 0.07)	32.1 ± 3.2	31.2 ± 3.6	0.2
BW / BL, %	1.6 ± 0.2*	11.0	-0.59 (p < 0.01)	1.7 ± 0.2*	1.6 ± 0.2*	0.28
Shorter spicule / longer spicule, %	37.2 ± 3.9	10.6	-0.19 (p = 0.08)	37 ± 4.2	37.4 ± 3.6	0.6

N o t e. Measurements in micrometres unless otherwise stated. CV — coefficient of variation, r_p — Pearson coefficient of correlation with body length.
*Distribution was not normal according to Shapiro-Wilk test.

Fig. 6. Males of *Icosiella neglecta*: A — general view; B — anterior part of body, left lateral view; C — posterior part of body, right lateral view; D — posterior part of body, ventral view; E — posterior end with shorter spicule on the right side of body, right lateral view; F — posterior end with shorter spicule on the left side of body, left lateral view; *oe-ij* — oesophago-intestinal junction, *ed* — ejaculatory duct.

head, short shaft, and spoon-shaped blade. Distal end of shorter spicule bifurcated, with two unequal tips. Smaller tip triangular, pointed. Larger tip thick, hook-shaped, directed ventrally (fig. 6, E, F). Longer spicule, when evaginated, resting along concave ventral side of shorter spicule's blade and between two tips of shorter spicule (fig. 6, D).

Small elevation of body wall present around cloacal aperture, forming anterior and posterior cloacal lips in lateral view (fig. 6, E, F). Caudal part of body short, rounded; muscle fibres present between dorsal side and posterior part of rectum. Slight constriction of body present on lateral sides just anterior to level of cloaca (fig. 6, D). Distance from cloacal aperture to tail tip 36–71.

Variability. Metrical characters in studied males of *I. neglecta* were in general less variable compared to those in females (table 4). Body length, body width at the border between the muscular and glandular oesophagus, length of the glandular oesophagus, and total length of the oesophagus showed the highest variability (CV > 20 %), while the other measurements were less variable. The lowest variability was observed in the length of spicules (CV < 10 %). Correlation between most direct measurements and body length was

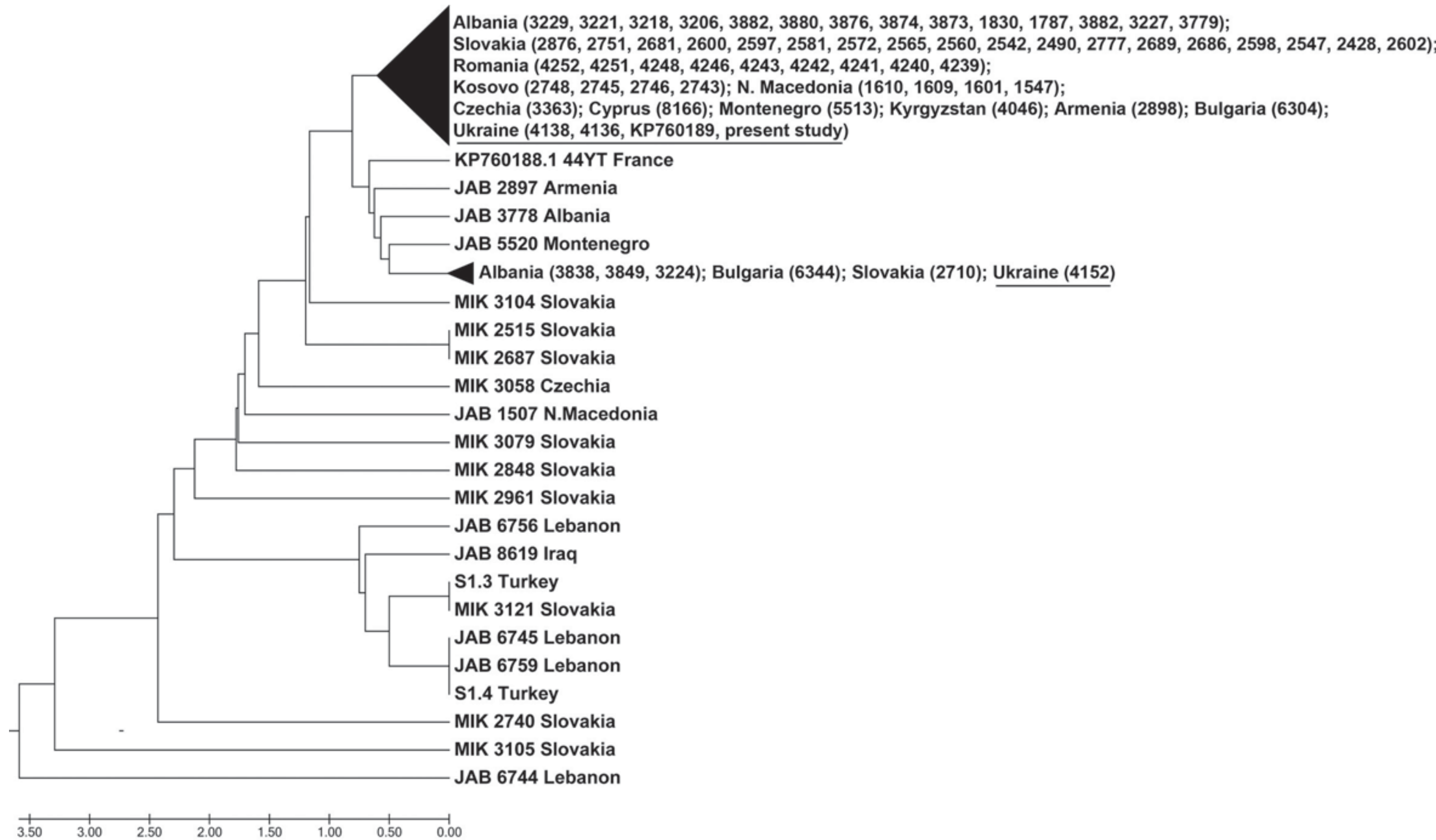

Fig. 7. UPGMA analysis based on 90 COI sequences of *Icosiella neglecta* from different localities. One sequence obtained in the present study, two sequences retrieved from Genbank (accession numbers KP760138 and KP760189) and 87 retrieved from the supplementary file to Mikulíček et al. (2021) following the original numbers.

rather high (r > 0.5) and significant (table 4). However, the correlation between the length of the longer spicule and body length was low (r = 0.29), while the correlation between the length of the shorter spicule and the body length was absent.

In the examined material, we distinguished two types of males in relation to the position of their spicules. In 43 males (54 %), the left spicule was long and thin, while the right spicule was short and robust. In 37 males (46 %), the right spicule was longer than the left spicule. In the subsamples of specimens from separate localities, the ratio of male types could differ. We observed 61 % (22 out of 36) males with a longer left spicule in a sample from Tyahle Lake, and 47 % (18 out of 38) of such males in a sample from Malyn. In both types of males, the shape of the corresponding type of spicule, either longer or shorter, was similar; they just differed in their position, either left or right (fig. 6, E, F). We found no statistical difference between the two types of males in their metrical characters (table 4). Moreover, body length, width of the muscular oesophagus at the isthmus, and the length of the shorter spicule were almost identical in the two groups of males (t-test p ≥ 0.9).

Microfilariae [12 specimens from the ovejector of one female were measured; measurements are given as the mean followed by the range in parentheses]. Body spindle-shaped, gradually narrowing from mid-region towards anterior and posterior end (fig. 5, F). Both extremities rounded. Sheath present. Body cuticle thin, finely transversely striated. Tooth small, pointed, directed almost anteriorly. Body filled with tightly-packed nuclei. Body length 89 (82–95), width at mid-length 6.5 (6–7).

5. Molecular characterisation of I. neglecta from Ukraine

In the present study, two of the most commonly used genetic markers (nuclear 18S and mitochondrial COI) newly generated from one specimen of *I. neglecta* collected in the city of Kyiv (Telbin Lake, locality no. 18) were used. Both obtained sequences appeared to be identical to the corresponding ones from *I. neglecta* specimens from Ukraine available in GenBank (accession numbers KP760138 and KP760189). Additionally, 87 COI sequences were retrieved from the supplementary file to Mikulíček et al. (2021) for comparison (fig. 7). The whole set included three sequences from western Ukraine (Zakarpatska Oblast): Ukraine 4136, 4138, and 4152 and one from the outskirts of Kyiv (KP760189). Most sequences from Ukraine appeared to be identical to each other, except for one from Zakarpatska Oblast (Ukraine 4152), which differed in one nucleotide. The UPGMA analysis showed that the COI sequence obtained in the present study was identical to 42 sequences of *I. neclecta* from Albania, Slovakia, Romania, Kosovo, Northern Macedonia, Czech Republic, Cyprus, Montenegro, Kyrgyzstan, Armenia as well as two sequences from western Ukraine and one from Kyiv (fig. 7). The different sequence from Zakarpatska Oblast was nested in the clade including identical sequences from Bulgaria, Slovakia and Albania.

Discussion

In the present study, we added a fifth COI sequence of *I. neglecta* from Ukraine to the previously published set of sequences from various parts of its distribution area. The new sequence appeared to be identical to three of four other sequences from the country and 42 sequences from other parts of the western Palaearctic, particularly, from Eastern Europe, Transcaucasia, and several Balkan countries. We assume that the population-genetic structure of *I. neglecta* in Ukraine is weak and not associated with the geography of the species populations within the country, similar to that discovered by Mikulíček et al. (2021) in the *I. neglecta* distribution area.

The present study did not cover the entire territory of Ukraine, as we did not collect any data from the western and eastern regions of the country (see fig. 1). However, *I. neglecta* was reported from those regions in previous surveys of helminths of amphibians (Mazurmovich, 1959, 1965; Shevchenko, 1966; Rezvantseva et al., 2010). We assume, therefore, that *I. neglecta* is distributed throughout the territory of Ukraine, similarly to its definitive hosts, the green frogs (*Pelophylax* spp.) (Pisanets, 2007). On the other hand, the nematode may be absent in some regions and frog populations due to the absence of its intermediate hosts and vectors, mainly *Forcipomyia velox* (Winnertz) (Ceratopogonidae) (Desportes, 1942). Mikulíček et al. (2021) suggested that the vectors of *I. neglecta* might have contributed to the relatively recent spread of the parasite. The recent spread of *I. neglecta* in northern Ukraine is apparent from the comparison of the information from previous surveys in this region with the results of the present study. In the outskirts of Kyiv, Mazurmovich (1951) found *I. neglecta* in 3 % of examined *P. ridibundus* and 8.8 % of *P. esculentus*, with a mean intensity of infection of 2.6 and a maximum of six nematodes in one frog. The author considered *I. neglecta* as one of the rarest parasites of green frogs in the region. In the present survey, the infection prevalence of *I. neglecta* in frogs from the city of Kyiv and Kyiv Oblast ranged from 20 % to 90 % (see table 1), and mean intensity reached 7.5 in some samples. Similarly, Maguza (1972) surveyed the helminths of amphibians in northern Ukraine and found *I. neglecta* in one out of 93 examined *P. esculentus* and in none of 61 *P. ridibindus*. We observed comparatively high *I. neglecta* infection parameters in the frog populations from Volyn, Rivne, Zhytomyr and Kyiv Oblasts; the infection prevalence reached 90–100 %, and the mean abundance and mean intensity of infection was above 10 in some examined samples (see table 1).

In the present study, we found no strong evidence of host preference of *I. neglecta* for either of the components of the *P. esculentus* complex. Infection prevalence was similar in the hybrids and the two parental species (see table 2). However, infection intensity in *P. ridibundus* was significantly higher than that in *P. lessonae* and *P. esculentus*. High infection intensity was also observed in the samples consisting of *P. ridibundus* or *P. ridibundus* + *P. esculentus*, though in the latter type of host populations the infection prevalence was comparatively low (40.5 %).

Infection intensity and abundance of *I. neglecta* were related to the body size of the hosts. Presumably, larger frogs offer more feeding sites for hematophagous vectors and/or accumulate parasites from consecutive infections. Higher correlation coefficient in the whole examined sample of hosts (including uninfected individuals) compared to that in the sample of infected hosts (0.57 vs. 0.47) suggests that mostly smaller frogs were uninfected. Besides, this positive correlation may be partly responsible for the higher infection intensity in *P. ridibundus* (SVL=72.4 ± 14.5 mm) when compared to *P. lessonae* (SVL=62.1 ± 6.5 mm). Similarly, females of *Pelophylax* spp. are larger than males and were found to harbour more specimens of *I. neglecta* in the studied samples. Interestingly, the prevalence of *I. neglecta* infection was slightly higher in males than in females. The reasons for such difference might be a subject of a separate investigation.

Most previous morphological descriptions of *I. neglecta* were based on small sample sizes; some of them included just one male and one female specimen. For example, Seurat (1917) gave a single value for each metrical character of males and females, except for body length in males (7.2–11.05 mm). Desportes (1941) complemented the description by Seurat (1917) by notes on the variability of body size in males and females, spicule size in males, and some qualitative characters. Besides, the author mentioned the variability in the position of the longer and shorter spicules in males of *I. neglecta*: the right spicule was longer and the left spicule was shorter in six out of seven examined males, while in one male, the left spicule was longer than the right one (Desportes, 1941). Enste (1954) briefly described *I. neglecta* from *P. esculentus* in Germany with no information on the variability, except for the range of female body length (6–36 mm). Maguza (1972) provided measurements of one male and one female of *I. neglecta* from Ukraine.

In the present study, we selected 40 females and 80 males of *I. neglecta* for morphological description and morphometry. We confirmed the diagnostic characters of *Icosiella* and *I. neglecta* distinguished by Seurat (1917) and Desportes (1941) in the morphology of the anterior extremity, digestive and reproductive systems: presence of four apical spikes, long glandular oesophagus, vulva at the anterior part of the glandular oesophagus, tail short and rounded in both sexes, spicules unequal and dissimilar, genital papillae in males absent. Metrical characters were found to be variable, especially in females; most of those variations were related to the body size of studied specimens. Correlations between various measurements and body length calculated in the present study may be useful in the identification and differentiation of *Icosiella* spp. The lowest variability was observed in the length of the spicules, especially the shorter one. In our opinion, this measurement may be diagnostic of *I. neglecta*. We did not confirm the observation of Desportes (1941) on the ratio of males with different positions of the long and short spicule. In the studied sample of 80 males, there were slightly more males (54 %) with a longer left spicule than with a longer right spicule, though in smaller samples the ratio could be different. Interestingly, Seurat (1917) described a longer left and shorter right spicule in *I. neglecta*, while in other descriptions, e.g. Dinnik (1930) and Enste (1954), the position of the longer and shorter spicules was not specified. A long and thin left spicule and short and robust right spicule is a typical pattern of most filarial nematodes (Bain et al., 2014). In *Icosiella*, some species were reported to have that typical position of spicules, e. g. *I. turgeocauda* Bursey, Telford et Goldberg, 2003, *I. intani* Purnomo et Bangs, 1996, and *I. hoogstraali* Schmidt et Kuntz, 1969; while in other species the right spicule was reported to be longer than the left one, e. g. *I. kobayasii* Yamaguti, 1941, *I. innominata* Yuen, 1962, and *I. laurenti* Bain et Purnomo, 1984 (Yamaguti, 1941; Yuen, 1962; Schmidt & Kuntz, 1969; Bain & Purnomo, 1984; Purnomo & Bangs, 1996; Bursey et al., 2003). To date, *I. neglecta* is the only known species of the genus with varying positions of longer and shorter spicules.

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