“BABEŞ-BOLYAI” UNIVERSITY
FACULTY OF BIOLOGY AND GEOLOGY
DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS-Summary

Conservative biogeography and the re-evaluation of the Pontic refuge in maintaining the genetic diversity of the species *Palingenia longicauda (OLIVIER, 1791)* (Ephemeroptera, Palingeniidae)

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General introduction

The genus *Palingenia* BURMEISTER, 1839, is a small but intensely debated genus of ephemeroptera, due to the controversial taxonomy of its species. The genus contains the largest extant ephemeroptera in the world, with body sizes between 25–40 mm and forewing span up to 30 mm. The genus contains the largest extant ephemeroptera in the world, with body size between 25–40 mm and forewing wing span up to 30 mm.

Within the genus, only four species have Palaearctic distribution (Demoulin, 1965; Bauernfeind and Soldán, 2012; Soldán, 2018): *P. anatolica* Jacob, 1977, *P. fuliginosa* (Georgi, 1802), *P. longicauda* (Olivier, 1791) and *P. sublongicauda* Tshernova, 1949. Data on the Afrotropic *P. apatris* (Demoulin, 1965), and the Indomalayan *P. orientalis* Chopra, 1927 are considered doubtful or insufficiently known (Bauernfeind and Soldán, 2012), as well as the locally distributed *P. anatolica* Jacob, 1977, which were described from the Asian part of Turkey by Jacob (1977), based on a single male individual.

Only three species have been recorded within the geographical limits of Europe so far. The type species of the genus, *Palingenia longicauda* (Olivier, 1791) is probably the best known mayfly ever, due to its impressive swarming and the short life-span of the adult.

Our research for the preparation of the doctoral thesis was carried out within the range of the Danube Delta Biosphere Reserve, within the range of the Prut river, within the range of the Mureș river, and within the range of the Bega river.

Based on a large sampling effort of *Palingenia longicauda* in its currently known range in southeastern Europe and in collaboration with researchers in Hungary, Ukraine, and Slovakia, we analysed several populations and compared with additional specimens, including *P. fuliginosa* and probably *P. sublongicauda* from Landa and Soldán's collection.

Aims of the study:

- Estimation of the current distribution area of the species on the territory of Romania and the detection of trends in the evolution of the population along the Danube and the main tributaries;
- Estimation of the genetic diversity of *Palingenia longicauda* populations of Romania, haplotype identification and comparison with populations from the Tisza basin;
- Re-examination of Soldán's original collection to clarify the presence of the three species of *Palingenia* in southeastern Europe, using integrative methods;
- Identification of cryptic populations applying the methodology *"citizen scince";*

- The reassessment of the protection status of the species *Palingenia longicauda* along the Danube and its inclusion in the National Red Lists, as well as the development of effective management for the sustainable conservation of this species.

Keywords: Conservation biogeography, rivers, *Palingenia longicauda,* integrative taxonomy, refuges, genetic diversity, Danube Delta, Prut river, Mureș river, services to society

Chapter I

The mayflies (Insecta: Ephemeroptera)

1.1. The origin and evolution of ephemeroptera

Ephemeroptera is an ancestral order of insects, dating back to the late Carboniferous, about 290 million years ago (Brittain and Sartori, 2003; Barber-James *et al.*, 2008). The Permian period confirms that the group was already present at the end of the Paleozoic (Sartori and Brittain, 2015). They are considered to have reached their maximum diversity in the Mesozoic (Brittain and Sartori, 2003), especially Jurassic and Cretaceous (Sartori and Brittain, 2015).

Ephemeroptera are considered to be the oldest and most primitive groups of insects existing today (Edmunds and McCafferty, 1988). Ephemeroptera are found in almost all fresh waters of the world, except Antarctica, the high Arctic region and some oceanic islands. A few species of South American Baëtidae are apparently semi-terrestrial (Brittain,1982).

1.2 Systematic classification of ephemeroptera

(Bauernfeind și Soldán 2012; http://www.faunaeur.org)

Kingdom Animalia
Subkingdom Eumetazoa
Phylum Arthropoda
Sub-phylum Hexapoda
Class Insecta
Order Ephemeroptera

1.3 Biology and ecology of ephemeroptera

In Bauernfeind and Soldán, 2012, ephemeroptera are described as hemimetabolous insects, which are characterized by a complex life cycle, consisting of an aquatic stage (egg, nymph) and aerial stage (sub-imago, imago). They are unique among insects in having two adult winged stages, sub-imago and imago (Brittain, 1982). Adults do not feed, but rely on reserves accumulated during their nymphal life. As adults, they generally live from 1 to 2 hours to several days, and spend most of their lives in the aquatic environment, either as eggs or as nymphs. The nymphal life span in ephemeroptera varies from 3 to 4 weeks to more than 2 years (Studemann et al., 1992; Sartori and Brittain, 2015).

Nymphs undergo a series of moultings as they grow, the precise number being variable within a species depending on external factors such as temperature, food availability and current speed (Brittain and Sartori, 2003). Between 10 and 50 moulting stages have been recorded (Ruffieux et al., 1996, Barber-James et al., 2008).

The nymphs, a stage very similar to the adults, result into the so-called sub-imago. There is a noticeable difference between adults, imago and sub-imago. The age of the sub-imago differs, depending on the gender, from a few minutes to 2-3 days. At the end of the period, it moults once more to become the imago, with a more vivid colouration and hyaline wings (Bogoescu, 1958; Sartori and Brittain, 2015).

Chapter II

Phylogeography and conservative biogeography of the species *Palingenia longicauda* (OLIVIER,1791)

***Case study* - An unexpected recovery of the long-tailed mayfly *Palingenia longicauda* (Olivier, 1791) (Ephemeroptera: Palingeniidae) in Southeastern Europe**

This chapter contains slightly modified parts of the following published article:

Avar L. DÉNES **· Romina M. VAIDA** · Emerencia SZABÓ · Alexander V. MARTYNOV · Éva VÁNCSA · Beáta UJVÁROSI · Lujza KERESZTES (2022) *Cryptic survival and an unexpected recovery of the long-tailed mayfly Palingenia longicauda (Olivier, 1791) (Ephemeroptera: Palingeniidae) in Southeastern Europe.* Journal of Insect Conservation, https://doi.org/10.1007/s10841-022-00425-z.

1. Introduction

Once widespread and well-known from the lower and middle courses of large and medium-sized rivers throughout Europe, by the second half of the 20th century *P. longicauda* was considered to be extinct in most of its historic range (Russev 1987; Soldán *et al.* 2009; Bauernfeind and Soldán 2012). For the previous decades *P. longicauda* was considered to be restricted only to the Tisza (or Tisa, Tysa) River and the lower range of its tributaries, and to the Rába (or Raab) river (Andrikovics et al. 1992; Kovács et al. 2001). This area corresponds to approximately 2% of its former range. In 2012, Bálint et al. published a comprehensive study that included 245 specimens from the extant populations of the Tisza river basin and the Rába river, which assessed the loss of genetic diversity that was caused by the large-scale range loss. Their results showed an unexpected high genetic diversity, and a significant genetic differentiation among populations from the Tisza river (228 specimens) and the Rába River (17 specimens). Those authors concluded that the species probably survived the last glacial maximum (LGM) in two medium Danube refuges, suggesting the possibility that the species persisted during the 20th century in the Rába river, in small and undetected populations.

In recent years several new reports about *P. longicauda* have been published, indicating the presence of the species in the Danube river in Hungary (Málnás et al. 2016), the Danube Delta in Romania (Soldán et al. 2009; Bulánková et al. 2013; Pavel et al. 2019) and Ukraine (Afanasyev et al. 2020), the Prut and the Dniester rivers in the Republic of Moldova (Munjiu 2018), and in the Styr river and the Horyn' river (i.e. the Pripyat river basin) in Ukraine (Martynov 2018 – as *Palingenia fuliginosa* (Georgi, 1802), misidentification).

In the context of these recently reported populations, we have focused on the identification of new potential habitats and the detection of *P. longicauda* larvae colonies on the major rivers of Romania. Our objectives were to assess the ecological conditions of the river sections where the species is present, the density (number of *P. longicauda* individuals per m^2) of the local populations, and the molecular genetic diversity of the species in the whole of the extended distribution area. Therefore, the present analysis focuses on two alternative hypotheses:

Hypothesis 1. The presence of the species in the lower sector of the Danube (including the Danube Delta and the rivers that are connected to it) is the result of recent recolonisation events from the already reported mid-Danubian refuges or the Tisza river basin. We predict that there is no genetic differentiation between populations of the Tisza or the Rába river systems and the studied populations from Southeastern Europe. We further predict that the genetic diversity of populations from the Danube Delta and the Prut river is considerably lower, due to the founder effect of the recent recolonisation.

Hypothesis 2. In contrast, the massive presence of the species in the Danube Delta and the Prut river represents overlooked populations, suggesting a recovery of some autochthonous populations. In this case, we predict a high genetic diversity and significant differentiation between the studied populations, similar to the pattern discovered by Bálint et al. (2012) between the Rába river and the Tisza catchment area.

2. Materials and methods

2.1 Sampling methods

Between 2018 and 2020, the presence of the species was identified and studied at 20 sites: the Mureș river (4 sites), the Prut river (6 sites), the Danube Delta (6 sites) in Romania; the Styr river

(3 sites), and the Horyn' river (1 site) in Ukraine (Fig.1). One adult individual was collected in Timişoara city, thus also indicating the presence of the species on the Bega river. Unfortunately, the location of the larvae colony was not yet identified, therefore, this river could not be included in any further analysis.

Where steep clay banks were identified, a 1 km-long sector of the bank was searched for evidence of larvae activity. Larvae were collected with an improved version of the "Bager" device, as described in the literature (Lengyel et al. 2004). This device was modified, in order to fit a semi-cylindrical shovel with a diameter of 25 cm and a depth of 30 cm, attached to a 2.5 m modular handle, which is similar to the tool that fishermen use on the Prut river (Fig.2). The riverbank was sampled three times at each collection site, by inserting the device to a maximum depth of around 2.5 m from the water surface. In this way, a 1 m^2 / 30 cm (approximately) section of the riverbed was extracted. The larvae were counted from each sample unit (three Bager samples per 1 m^2). The condition of the colonies was estimated, based on the average number of larvae in each sampling unit, and the abundance was estimated based on the metrics that have been recommended by Russev (1987) and Lengyel *et al.* (2004).

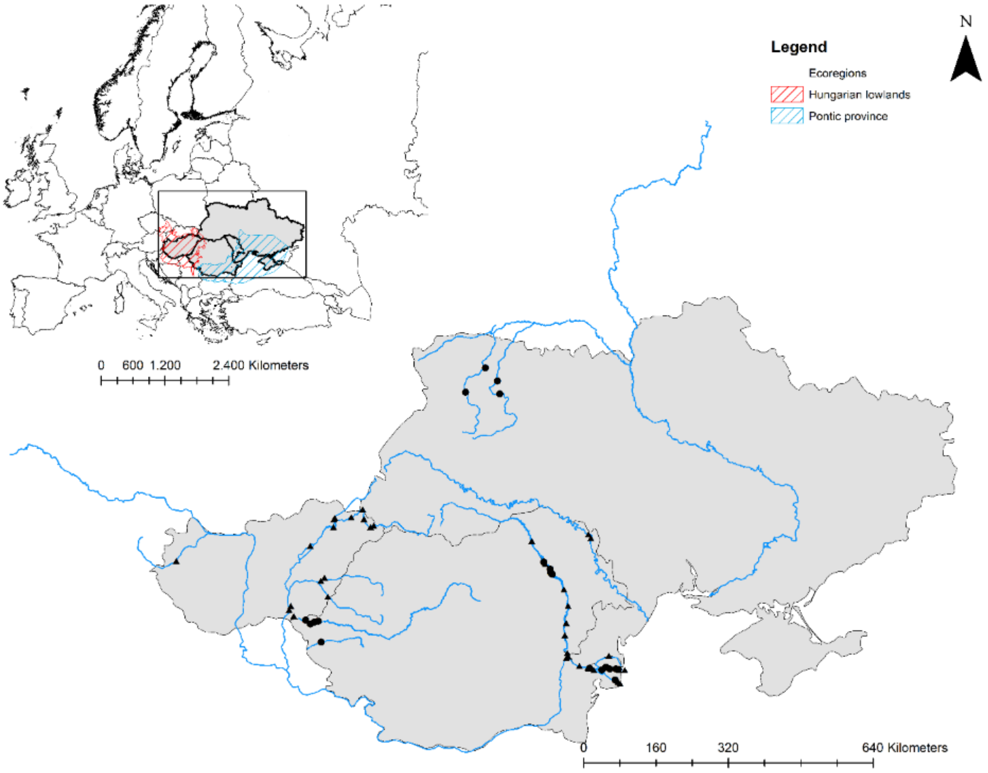

Fig. 1 Map showing the distribution *P. longicauda*.

Dots stand for sites identified by this study in Romania (Mureş, Danube Delta, Prut and Bega rivers) and Ukraine (Styr and Horyn'). Triangles show reported presence by the literature in Hungary (Rába River and Tisza River basin – Bálint et al. 2012), in Republic of Moldova (Prut and Dniester rivers – Munjiu 2018) and in Romania (Danube Delta – Pavel et al. 2019). The small map shows the Hungarian lowlands and the Pontic province ecoregions according to the European Union (EU) ecoregions for rivers and lakes (European Environment Agency, https://www.eea.europa.eu/data-and-maps/figures/ecoregions-for-rivers-and-lakes.

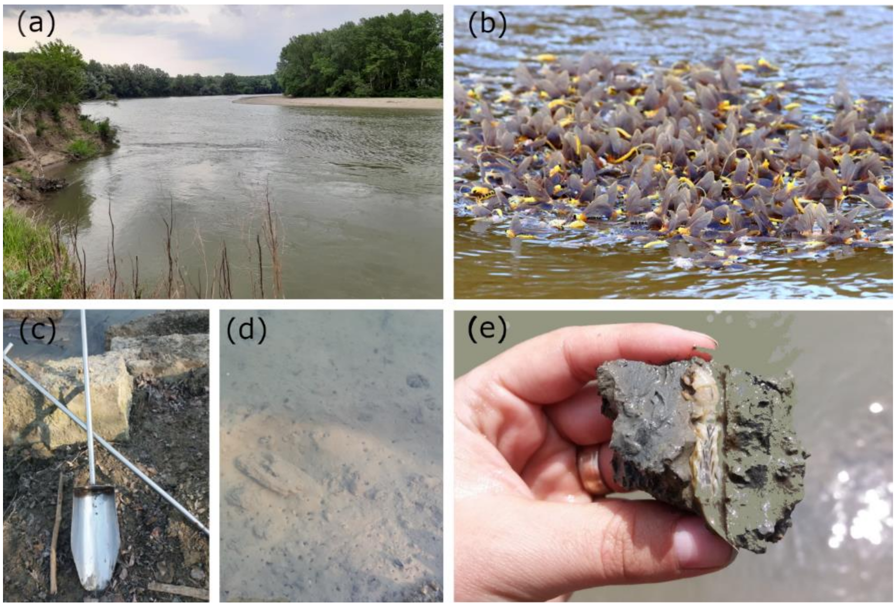

Fig. 2 **(a)** Typical *P. longicauda* habitat with steep clay banks (Mureș River, Nădlac, Arad county, photo: Vaida R.); (b) group of individuals during the mass swarming (Danube Delta, Maliuc, Tulcea county, photo: Petrescu D.); (c) modified "Bager" device; (d) the openings of the horizontal U-shaped borrows made by the larvae (Prut River, Iași county, photo: Vaida R.); (e) larva in the burrow (Mureș River, Nădlac, Arad county, photo: Vaida R.).

2.2 DNA sequencing

We considered that all individuals which were collected from a river belong to the same population, therefore, we did not include specimens from each collection site in the genetic analysis (Table 1). The individuals collected from the Styr river (7 specimens) and the Horyn river (5 specimens) were grouped together in the analysis as the Pripyat river basin.

Genomic DNA was extracted from 196 specimens, using the ISOLATE II Genomic DNA Kit (Bioline Meridian Bioscience, Inc. Cincinnati, OH, USA). To be able to integrate the sequence data of the Hungarian populations (Bálint et al. 2012), a 471 base pairs (bp) section of the *mt*COI gene and a 464 bp fragment of the *mt*16S LSU were amplified. The *mt*COI sequences were amplified using the Jerry (Simons et al. 1994) – S20 (Pauls et al. 2006) primer pair. The 16Sar (Simons et al. 1994) – 16SB2 (Monaghan et al. 2007) primer pair was used for the amplification of *mt*16S LSU. PCR reactions were carried out in a 25 µl reaction volume containing 0.5 µl MyTaq™ DNA Polymerase (Bioline Reagents Ltd., London, UK), 5 µl 5x MyTaq™ Reaction Buffer, 1 µl of the primer pair mix (20 µM each), 1 µl of the template DNA, and PCR-grade water (Jena Bioscience, Jena, Germany) up to 25 µl. An annealing temperature of 40 °C was set for the *mt*COI, and of 56 °C for the mt16S LSU fragments. The PCR products were loaded onto a 1% agarose gel and the target fragments were cut out and purified using a Wizard SV Gel and PCR Clean–Up System (Promega, USA). The purified PCR products were sent to Macrogen Europe

(Amsterdam, Netherlands) for sequencing. The resulting sequences were verified at the NCBI website using a Basic Local Alignment Search Tool (BLAST) (Johnson et al. 2008). Sequences were aligned in BioEdit version 7 (Hall, 1999) using the Clustal W multiple alignment algorithm, and the concatenation was undertaken manually. Consensus sequences were deposited in GenBank (accession numbers, *mt*COI: MW716042 – MW716237; *mt*16S LSU: MW717693 – MW717888).

2.3 Estimating genetic diversity

The number of haplotypes and polymorphic sites (S), the haplotype (Hd), nucleotide diversity (π) of the *mt*COI, the *mt*16S LSU, and the concatenated data sets were calculated in DnaSp 6 (Rozas et al. 2017). Genetic diversity indices were calculated separately for the dataset that was generated by the present study, and by Bálint et al. (2012), as well as for the combined datasets. Genetic diversity was also estimated for sequences that were grouped, based on the studied rivers.

2.4 Tests for differentiation among populations

The *mt*COI and *mt*16S LSU datasets were checked against conflicting phylogenetic information, based on the topology of the Neighbour-Joining trees, that were generated using 10,000 bootstrap replicates in Mega X (Kumar et al. 2018). Phylogenies were further estimated for the *mt*COI, *mt*16S LSU, and the concatenated dataset using a Median-Joining (MJ) haplotype network that was implemented in PopArt 1.7 (Leigh and Bryant 2015).

The genetic differentiation among populations representing different studied rivers was estimated using an exact test of population differentiation (ETPD) based on haplotype frequencies (Raymond and Rousset 1995), and with the pairwise F_{ST} values using Arlequin 3.5 (Excoffier and Lischer 2010). The genetic differentiation was further assessed using the hierarchical analysis of the molecular variance (AMOVA), which was implemented in Arlequin 3.5 (Excoffier and Lischer 2010).

2.5 Mismatch distributions and tests of selective neutrality

Mismatch distributions were calculated in order to identify patterns of historic demography for the populations of the studied rivers. Calculations were performed using the concatenated dataset in Arlequin 3.5 (Excoffier and Lischer 2010) under a model of sudden expansion, with 10,000 bootstrap replicates. A unimodal distribution shows that a lineage has undergone recent population expansion, while a multimodal distribution suggests a constant population size or geographical subdivision (Marjoram and Donnelly 1994). The appropriateness of this model was evaluated by using the sum of squared deviations (SSD) and Harpending's raggedness index (RI) (Harpending 1994). Tajima's *D* index (Tajima 1989), and Fu's *F*s test (Fu 1997) were also calculated using Arlequin 3.5 (Excoffier and Lischer 2010), with 10,000 simulated samples. The two tests are frequently used in combination with mismatch distributions in order to indicate recent demographic expansion.

3. Results

3.1 New populations of P. longicauda in Southeastern Europe

On the Mureș river (Romania), larvae colonies were first identified near the western limit of Arad city, and they were continuously sampled on the clay bank of the river towards Nădlac (the area of the Lunca Mureșului Natural Park (Romania), with a total length of approx. 88 km, and investigated using a boat). The average number of larvae was high, 83.25 individuals/m^2 (N=36 sample), which represents a high density and a "good" condition of the colonies, according to Russev (1987) and Lengyel et al. (2004) (Table 2). The number of larvae was variable among sites, from 69 individuals/m^2 (Șemlac, Romania) up to 99 individuals/m^2 (near Nădlac, Romania).

In the case of the Prut river, which represents the border between Romania and the Republic of Moldova, we searched the riverbank on the Romanian side. A 60-km section of the river was searched between the localities Șendreni and Țuțora, and five sites with near-natural habitat conditions were sampled. Larvae colonies were present and eudominant in the clay bottom of every site, with an average number of 69.6 individuals/m^2 (N=45 sample). The colonies showed similar conditions to those from the Mureș River, with high individual densities on every location. However, important differences were observed in the numbers of individuals among different collection sites, ranging from 50 individuals/m^2 (Șendreni) up to 108 individuals/m^2 (Țuțora), without any apparent anthropogenic modification of the riverbank.

3.2 Estimates of genetic diversity

We generated 196 sequences for both *mt*COI and *mt*16S LSU markers, representing populations from Romania and Ukraine. Additionally, 245 sequences were downloaded for each marker, representing the Hungarian populations. Only one individual was collected from the Bega River and corresponded to haplotype H3. This river was not used in any further analysis due to the lack of information. The two rivers from Ukraine, the Styr river (7 individuals) and the Horyn' river (5 individuals), were grouped together as tributaries of the Pripyat river.

Results of the genetic diversity estimations are summarised in Table 3. Calculations showed high haplotype and low nucleotide diversity for each dataset. The sequences from the study by Bálint et al. (2012) showed 31 haplotypes for the *mt*COI, 53 for the *mt*16S LSU, and 87 for the concatenated alignment. Sequences generated by the present study showed 32 *mt*COI haplotypes, 42 *mt*16S LSU, and 75 haplotypes for the concatenated dataset. The combined datasets of 441 sequences for each marker showed 57 *mt*COI haplotypes (6 present in both datasets), 86 *mt*16S LSU haplotypes (9 shared by both datasets), and 148 haplotypes (14 shared between the two datasets) for the concatenated markers. Genetic diversity estimates showed similar results when sequences were grouped based on the rivers. Haplotype diversity ranged from 0.67 to 0.81 for the *mt*COI sequences, from 0.45 to 0.81 for the *mt*16S LSU sequences, and from 0.76 to 0.93 for the concatenated dataset. Geographic distribution of haplotypes is summarised in the phylogenetic network (Fig.3).

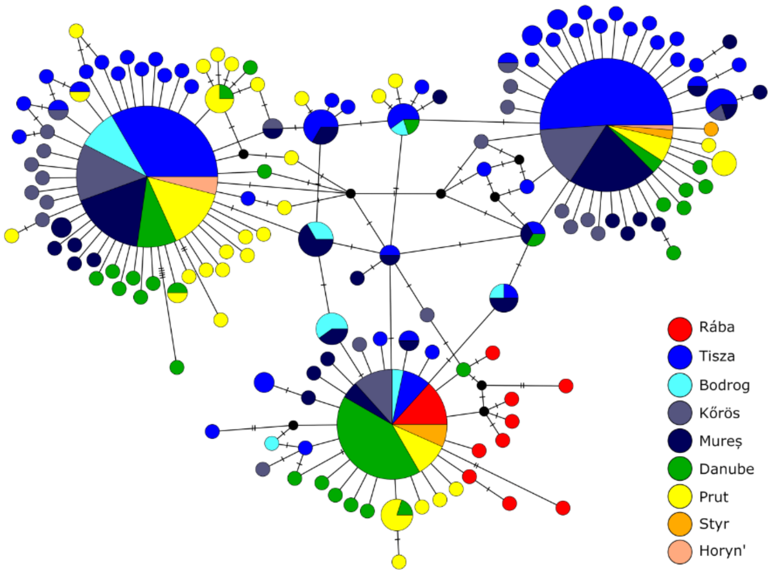

Fig.3 Median-Joining haplotype network generated for the concatenated dataset using PopArt 1.7. Each circle represents a unique haplotype and circle size is proportional to the number of samples observed for that haplotype. The number of mutations is represented by hatch marks on the lines. Colours correspond to different rivers. H1, H2, and H3 correspond to the three major haplotypes discussed in the text.

3.3 Population structure and patterns of diversity

The Neighbour-Joining trees showed similar results for both the *mt*COI and the *mt*16S LSU sequence alignments, and for the concatenated dataset. As no conflicting phylogenetic information was observed, and the tree topologies were similar to those shown by the MJ network analysis, these data have not been shown or discussed further (Fig. 3, Fig. S1).

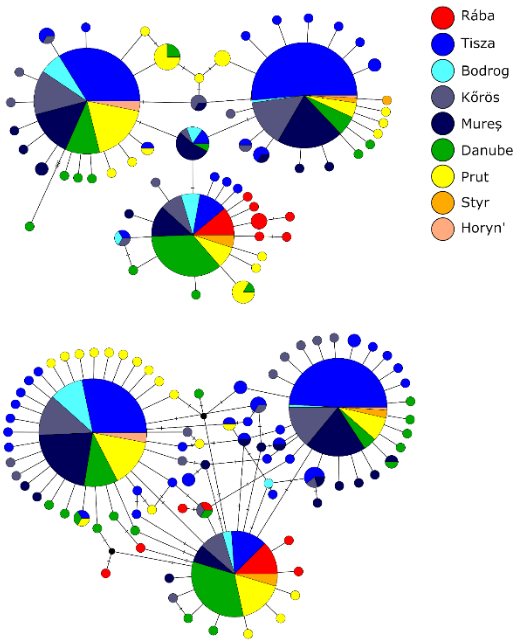

Fig. S1 Median-Joining haplotype network generated for the *mt*COI (top) and *mt*16S LSU (bottom) sequence alignments using PopArt 1.7.

The MJ networks had similar star-like topologies for both markers separately, and also for the concatenated dataset (Fig. 3), therefore, only the latter was further discussed. The network shows no geographic structure in haplotype distribution. Three frequent haplotypes dominate the network (hereinafter, referred to as H1, H2, and H3), represented by individuals from almost every river (Fig.3). Of these common haplotypes, only H1 was found in the Rába river population. The Tisza and its tributaries (the Bodrog river, the two Kőrös rivers, and the Mureș river) share two additional haplotypes with the Danube Delta, and one with the Prut river, besides the three common ones. The Danube Delta and Prut river populations have three additional shared haplotypes (Fig.3). The majority of the identified haplotypes were restricted to only one of the studied rivers. The specimens that were collected from the Bodrog river represented 7 haplotypes, out of which only 1 was private. In the Pripyat river basin, out of 4 haplotypes 1 was private. In contrast, out of the 10 haplotypes that were identified in the Rába river population, only 1 was shared and the other 9 were endemic. The private haplotype-count in other rivers ranged from 55.17% of the total number of haplotypes, to 78.12%, with an average of 69.92%.

The population pairwise F_{ST} calculations showed statistically significant differentiation between the Rába river and populations present on each studied river, with an average of $F_{ST} = 0.310$ (Tabel 1). The lowest pairwise difference was shown between the Rába river and the Danube Delta ($F_{ST} = 0.155$, $p < 0.001$). The ETPD shows no differentiation between populations of the two rivers (p = 0,19; Table 1). The F_{ST} values were statistically significant when the Tisza river was compared with the Danube Delta ($F_{ST} = 0.128$; $p < 0.001$) and the Prut river ($F_{ST} = 0.111$; $p < 0.001$). These differences were also supported by the statistically significant differentiation values ($p < 0.001$) of the ETPD (Table 1). A lower, but also statistically significant difference was observed between the Danube Delta and the Prut river ($F_{ST} = 0.064$, $p < 0.001$), with strong support for differentiation at $p < 0.001$ (Table 1).

The analysis of molecular variance showed that most of the variance was found within individual collection sites (89.20%, $F_{ST} = 0.108$, $p < 0.001$), followed by the variance among populations from different rivers (7.85%, $F_{CT} = 0.078$, $p < 0.001$). The lowest variation was found among collection sites within the different rivers (2.95%, $F_{SC} = 0.032$, $p < 0.05$). Analysis of molecular variance (AMOVA) showed that there is a strong genetic structure in two of the tested levels of structural hierarchy (within collection sites: $F_{ST} = 0.108$, $p < 0.001$; among rivers: $F_{CT} = 0.078$, $p < 0.001$). In these cases, the null hypothesis of no differentiation can thus be rejected.

Tab.1 Genetic differentiation of populations from the different rivers. Pairwise *Fst* values (lower left) and significant ETPD (upper right) results of extant populations. Bold values are significant at: **<0.001 and **<0.01.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
(1) Rába		$+^{**}$	$+^{**}$	$+^{**}$	$+^{**}$	-	$+^{*}$	-
(2) Tisa	**0.372****		-	-	-	$+^{**}$	$+^{**}$	-
(3) Bodrog	**0.382****	**0.174****		-	-	-	-	-
(4) Körös	**0.333****	0.002	**0.107***		-	$+^{**}$	-	-
(5) Mureș	**0.346****	0.001	**0.117***	-0.009		$+^{**}$	$+^{**}$	-
(6) Delta Dunării	**0.155****	**0.128****	0.060	**0.073***	**0.082****		$+^{**}$	-
(7) Prut	**0.312****	**0.111****	0.022	**0.054***	**0.069****	**0.064****		-
(8) bazin Pripyat	**0.276****	0.006	0.089	-0.028	-0.022	-0.011	0.026	

3.4 Mismatch distributions and tests of selective neutrality

The analysis of the demographic history for the whole dataset shows significant departure from the equilibrium. Both Tajima's D index and Fu's Fs test showed negative values with significant support (Tajima's $D = -2.393$, $p < 0.001$; Fu's $Fs = -25.762$, $p < 0.001$), and the mismatch distribution plot (Fig. S2) fits well with the sudden population expansion model (SSD = 0.0119, $p = 0.062$; Raggedness index = 0.028, $p = 0.16$). Similar results were observed on a regional scale. Tajima's D and Fu's Fs showed negative values and significant departure from equilibrium for each population, except for the Bodrog river and the Pripyat tributaries (Table 2). Significantly negative values indicate a recent demographic expansion. The mismatch distribution plots show unimodal distribution, and together with SSD and Raggedness index values support a recent demographic expansion for populations of each river, except for the Pripyat tributaries (Table 2, Fig. S2).

Tab.2 Results of mismatch distribution and neutrality tests for the whole dataset, the two lineages identified by BAPS, and for populations from different rivers.

	Mismatch distribution				**Test of selective neutrality**			
	SSD	***p***	**RI**	***p***	**Tajima's D**	***p***	**Fu's Fs**	***p***
All	0.011	0.062	0.028	0.162	-2.393	0.000	-25.762	0.000
Rába	0.049	0.078	0.199	0.046	-1.818	0.021	-5.399	0.0008
Tisa	0.015	0.290	0.032	0.461	-1.801	0.008	-26.433	0.000
Bodrog	0.006	0.736	0.029	0.908	-0.258	0.442	-1.556	0.152
Körös	0.021	0.093	0.047	0.191	-1.515	0.044	-15.278	0.000
Mureș	0.015	0.115	0.041	0.183	-1.335	0.068	-23.833	0.000
Delta	0.016	0.317	0.034	0.452	-1.938	0.008	-23.071	0.000
Prut	0.008	0.054	0.026	0.199	-1.829	0.012	-25.914	0.000
Pripyat	0.120	0.009	0.284	0.043	1.159	0.880	1.578	0.809

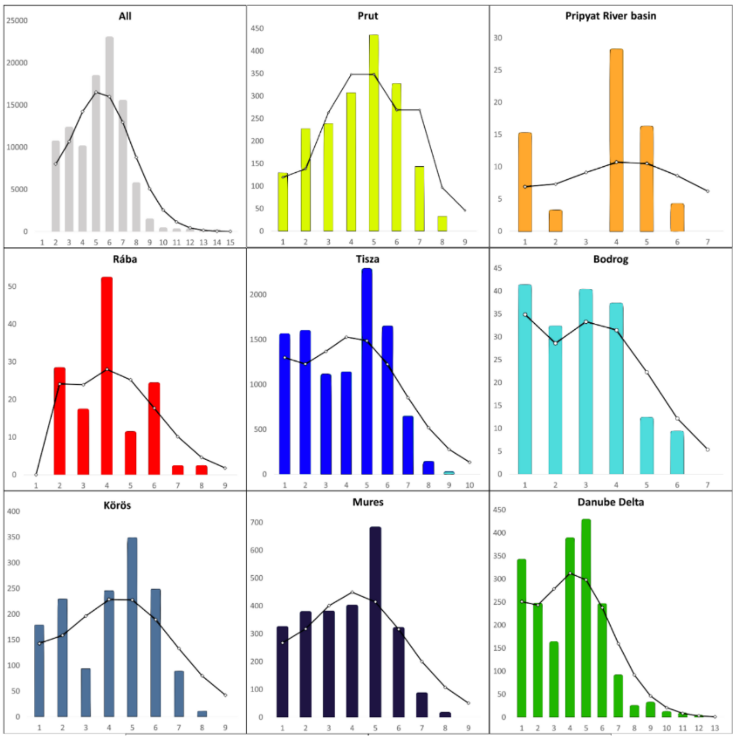

Fig. S2 Mismatch distribution histograms, for the whole dataset (All) and for populations from each river. Bars indicate the observed values and black lines show the expected distribution under the sudden expansion model.

4. Discussion

4.1 Genetic diversity of P. longicauda

The extension of the molecular analyses to the wider distribution area of *P. longicauda* allowed us to have a more comprehensive insight into the genetic structure of the species. The two studied mitochondrial markers show high haplotype diversity and low nucleotide diversity for the species. This pattern was observed in each studied population from Romania and Ukraine, which is similar to the pattern that was observed for the populations from Hungary (Bálint et al. 2012).

The individuals that were collected from the Styr river (7 specimens) and Horyn' River (5 specimens) were grouped together in the analysis as the Pripyat river basin. These samples were recorded as *P. fuliginosa* by Martynov (2018), based on a morphological study of larvae and subimagoes. DNA material of these specimens was originally planned to be used as an out-group species in the present study, but investigation of their mitochondrial sequences revealed that they belong to *P. longicauda.* This misidentification was later confirmed by an investigation of male imago genitalia. The Pripyat river basin group showed no statistically significant differentiation from any of the studied rivers. This is likely due to the low sample size and the high frequency of the three common haplotypes, and shows the need of a more intensive sampling and population genetic study with a focus on this region.

The results suggest that there is significant genetic structure in the concatenated dataset of the two studied markers. Calculations of pairwise differentiations indicate statistically significant differences among the Danube Delta, the Prut river, the Rába river, and the Tisza river populations. The lowest statistically significant value of pairwise differentiation was shown between the Rába River and the Danube Delta populations. In this case, the ETPD did not confirm the differentiation. These results were further confirmed by the AMOVA, which showed a strong genetic structure at the highest tested levels of structural hierarchy (among rivers: $F_{CT} = 0.078$, $p < 0.001$).

Three haplotypes (H1, H2, and H3) represented 56.13% of the total individuals, and were commonly present in almost every river. The majority of the other haplotypes were endemic to the different studied rivers (Fig.3). The average endemic haplotype frequency in rivers was 59.86%, ranging from 25% to 90%. Sixty-one (61) haplotypes were identified in the lower-Danube region, however, besides the three frequent haplotypes (H1 – H3), only three additional haplotypes (H111, H120 and H121) were shared by the Danube Delta and the Prut river populations (Fig.3).

In the previous study focusing on the genetic diversity of *P. longicauda*, Bálint et al. (2012) established that the Tisza catchment populations and the Rába population are differentiated, identifying two important regions for the conservation of the species, suggesting that the mitochondrial mutation rates which were observed in other insects could not have led to the accumulation of enough genetic signal in the past 50-60 years to result in the identified divergence.

The high number of endemic haplotypes, and the statistically significant differentiation found between the populations from the Danube Delta and the Prut river is in line with their findings, confirming that the populations of these two rivers also had independent histories.

The high haplotype diversity and low nucleotide diversity, coupled with the high number of private, endemic haplotypes can suggest a population growth after a period of low effective population size (Grant and Bowen 1998). This is also supported by the statistically significant and negative Tajima's D and Fu's *F*s results (Alcaraz and Gholami 2020; Ivanova 2021). These findings are in alignment with Hypothesis 2, and show that the individuals collected on the Danube (in the Danube Delta) and the Prut rivers, represent overlooked local populations. Our study, therefore, identifies two additional river regions that can greatly contribute to conservation efforts of *P. longicauda*, and confirms the survival and recovery of this species in Southeastern Europe.

The middle-Danube region (the Rába and the Tisza river basin) and the lower sector of the Danube river (the Danube Delta and the Prut River) share only three other haplotypes (H20, H38 and H39) besides the three common haplotypes (Table S2). The species is considered to be a Pontic biogeographical element (Haybach 1998), and this region is accepted as an important diversification centre and refuge area for many freshwater species (e.g. Bănăduc et al. 2016; Bauernfeind and Soldán 2012; Csapó et al. 2020).

The post-glacial upstream recolonisation of Northwestern Europe through the Danube basin is a well-established paradigm of the freshwater zoogeography (Bănărescu 1991; Varga 2010). We can, therefore, assume that the three frequent, major haplotypes that are present in almost every river of this study, could reflect a founder effect of an upstream colonisation, with all three reaching the Pannonian basin.

The population from the Rába river represents the westernmost known extant distribution of the species. Individuals from this river correspond exclusively to H1 and to private haplotypes linked to it, showing that H1 could have migrated further upstream, reaching western parts of Europe. This is also confirmed by Bálint et al. (2012), who found no genetic differences between the Rába and the extinct Rhine populations, based on the analysis of a 196 bp mitochondrial sequence of the museum specimens and the extant populations.

The haplotype distribution does not show any clear differentiation pattern between the middle-Danube region (Pannonian region) and the lower-Danube (Pontic region), based on the two studied markers. However, the high number of endemic haplotypes and the low number of shared haplotypes, together with the statistically significant differentiation that is observed among the rivers of the two regions, indicate the need of a more comprehensive study, relying on a higher number of genetic markers, or on next generation sequencing techniques, in order to better understand the phylogeographic history of *P longicauda*.

The presence of the species in the two Ukrainian rivers (the Styr river and the Horyn’ river) can also be explained by a colonisation from the Pontic region. The Pripyat river is a tributary of the Dnieper (or Dnipro) river, which, together with the Southern Buh river (or Pivdennyi Buh) and the Dniester river (or the Dnister), is also recognised as a migration corridor for aquatic biota from the Black Sea coast to Northwestern Europe (Bij de Vaate et al. 2002; Jażdżewska et al. 2020; Sworobowicz et al. 2020). The presence of the species in this region indicates the need for a more focused search for it along this central migration corridor.

5. Conclusions

This is the first molecular genetic study of the recently discovered populations from Southeastern Europe. This work confirms the previously reported presence of *P. longicauda* populations in the Danube Delta and on the Prut river, and shows the presence of the species on the Mureș River in Romania. It also shows the presence of the species on the Bega river in Romania and on the Styr river and the Horyn’ river in Ukraine, although these locations do require further investigations. The results, that are based on the analysed mitochondrial DNA markers (*mt*COI and *mt*16S LSU), indicate that the populations on the Prut River and Danube river (in the Danube Delta) are well differentiated from the Tisza river basin populations. The large number of endemic haplotypes and the statistically significant differentiations suggest that these are recovered local populations, which have been overlooked in the past decades. These populations, therefore, can provide an important contribution to the long-term survival and sustainable conservation of the species in this part of Europe.

Based on the field observations, the presence of the species is closely connected to near-natural river sections in Romania, where the hydromorphological integrity of the riverbed was not disturbed. Important larvae colonies were only detected on steeply-inclined clay riverbanks with a constant flow of water.

Chapter III

Taxonomic revision of the species *Palingenia longicauda* (OLIVIER,1791);

***Case study*:- Dilemma lasting decades solved? Integrative taxonomy supports one instead more *Palingeni*a species in South-Eastern Europe (Insecta, Ephemeroptera, Palingeniidae)**

[2]This chapter contains slightly modified parts of the following published article:

Peter Manko[1], **Romina Mirabela Vaida[2,] ***, Lujza Keresztes[2], Alexander Martynov[3], Emerencia Szabó[2], Beáta Baranová[1], Béla Kis[4], Éva Váncsa[5], Avar-Lehel Dénes[6] - *Integrative taxonomy supports one rather than several species of Palingenia in South-Eastern Europe (Insecta, Ephemeroptera, Palingeniidae)- The European Zoological Journal* 296-306, Vol.90, No.1; https://doi.org/10.1080/24750263.2023.2191622

1. Introduction

Palingenia Burmeister, 1839, is a small genus of the mayfly family *Palingeniidae* (Insecta, Ephemeroptera) that have an important conservation value for the ecological integrity of large rivers (Bálint *et all.*, 2012). The genus contains the largest extant mayflies in the world, ranging in body size between 25–40 mm, and with the forewings span up to 30 mm, and their major morphological characters were recently summarized by Bauernfeind and Soldán (2012).

Only four species have Palearctic distribution (Demoulin 1965; Bauernfeind & Soldán 2012; Soldán & Landa 1986): *P. anatolica* Jacob, 1977, *P. fuliginosa* (Georgi, 1802), *P. longicauda* (Olivier, 1791) and *P. sublongicauda* Tshernova, 1949. Data on the Afrotropic *P. apatris* (Demoulin, 1965), and the Indomalayan *P. orientalis* Chopra, 1927, are considered doubtful or insufficiently known (Bauernfeind & Soldán 2012).

Three species have been recorded within the geographical boundaries of Europe so far. The type species of the genus, *P. longicauda* (Olivier, 1791), is probably the best known mayfly, because of its large mating/swarming flight and short adult lifespan; it was probably the first mayfly ever noticed by man (Russev 1987; Soldán 1997; Haybach 2007). The second European species of the genus, *P. fuliginosa* (Gregori, 1802), was named and described by Boeber and noticed by Gregori in 1802 from the Caucasus (see references in Bauernfeind & Soldán 2012), but also from Azerbaijan, Russia and Iran (Kasymov & Agaev 1986). The species has a controversial taxonomical history, as it was considered a junior synonym of *P. longicauda* for a long time but was redescribed by Tshernova (1949) from the southern part of European Russia. It was also surprisingly mentioned by Landa (1969) and Landa & Soldán (1985) in sympatry with *P. longicauda* from the lower Latorica and upper Bodrog Rivers (Tisa River basin) in Slovakia.

Later, Godunko & Kłonowska-Olejnik (2003) also recorded some individuals from the Latorica (Latorca) River in Ukraine. Soldán (1978) published a general revision of the European species of *Palingenia*, and it contained the most comprehensive identification keys to discriminate between the three European *Palingenia* species, based mainly on the Slovak populations (except for *P. sublongicauda*, which were most probably obtained by loan from Tshernova, from southern Russia). The third European species, *P. sublongicauda* Tshernova, 1949, was described from the southern part of Russia (Tshernova 1949) and keyed by Soldán (1978).

The objective of the present paper was to re-examine the original collection of Soldán (Figure 1) in order to clarify the presence of these three *Palingenia* species in south-eastern Europe. We used quantitative morphology and mitochondrial DNA sequences to test taxonomy hypotheses of the species based on integrative data and review the species distribution based on newly detected populations from south-eastern Europe.

Fig. 4. Soldán's and Landa's reference collection of European *Palingenia* from the 1970s, deposited in the Institute of Entomology, Prague, Czech Republic.

2. Material and methods

2.1. Sampling and data collection

The type material of the three species (*P. fuliginosa*, *P. longicauda* and *P. sublongicauda*) recorded from Europe were not available during our investigations. However, the presence of *P. fuliginosa* in the studied area was reported by Soldán and Landa; therefore, we re-examined their reference collection of European *Palingenia* from the 1970s, deposited in the Institute of Entomology, Biology Centre CAS, České Budějovice, Czech Republic (Fig.4). The collection contains hundreds of individuals of *Palingenia* labelled as *P. longicauda* and *P. fuliginosa.* Unfortunately, the collection does not contain individuals marked as *P. sublongicauda*, despite the fact that Soldán seems to have worked with individuals identified as *P. sublongicauda* in his revision (referring to 6 larvae, 3 males, 1 female and 1 sub-imago from the Volga river, from 1935, without any collection data) (Soldán, 1978).

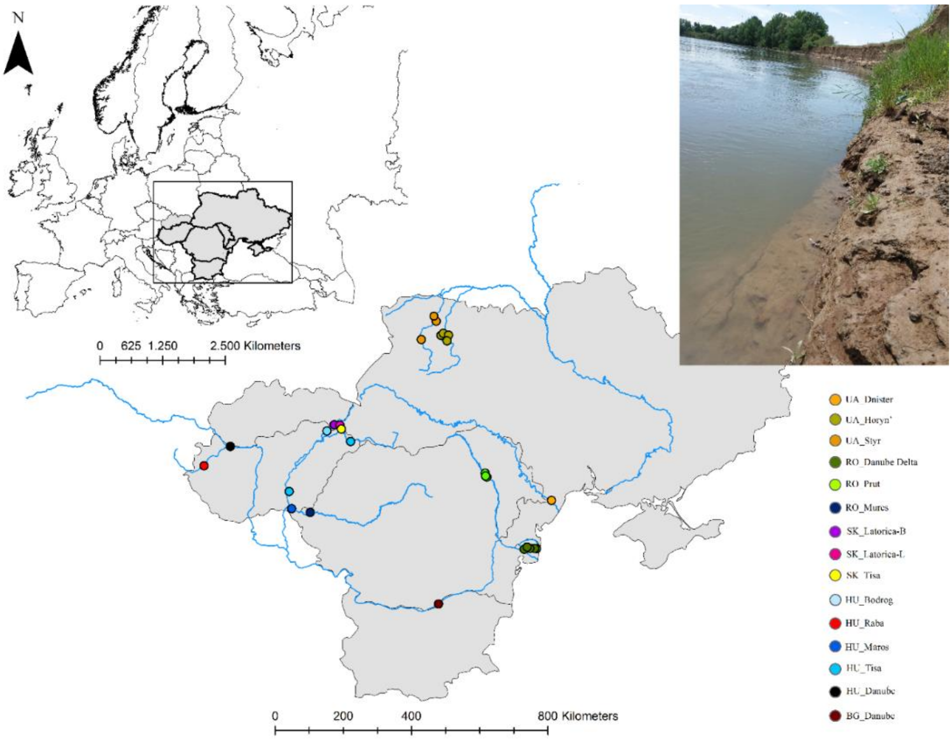

Fig. 5. Map showing the distribution of the analysed sampling locations. The photo in the upper right corner shows a typical *P. longicauda* habitat with steep clay banks (Mureş River, Nădlac, Arad county, photo: Vaida R.).

In our morphological revision, 174 specimens of male *Palingenia* were analysed from 11 different sampling locations, representing 7 rivers (Fig. 5). The analysed individuals from Soldan's collection represented populations from the Latorica (Slovakia), the Tisa (Slovakia) and the Danube (Bulgaria). The fresh *Palingenia* material was collected from the Danube (Hungary and Romania), the Latorica (Slovakia), the Tisa (Hungarian-Slovak border), the Mureș (Romania), the Prut (Romania), the Horyn' (Ukraine) and the Dniester (Ukraine). Molecular analysis included larvae material from 5 additional rivers: the Raba river (Hungary), the Tisa (Hungary), the Bodrog (Hungary), the Maros (Hungary) and the Styr (Ukraine) (Fig.5).

2.2 Morphometry and statistical analyses

Linear morphometry was applied to quantify the possible morphological variability among the populations. In particular, the ratios of the characters were calculated as also used in Soldán's (1978) revision and are invariant for a particular measure of size (Mosimann 1970).

The morphometric characters analysed in this study included the ratio between size and distance of the compound eyes, the ratio between the penis lobe length and the distance between penis lobe tips, the ratio between the penis lobe length and the base width, as well as the penis lobe angle were calculated to verify the differences described by Soldán (1978). Measurements were made using photographs taken with a stereomicroscope equipped with a digital camera. Subsequent image analyses (measurements) of the obtained photographs were performed in ImageJ (ver. 1.53k, Schneider *et al.* 2012), as shown in Fig.6, and then the given ratios were calculated. Basic summary (univariate) statistical analyses of morphological measurements (Median, Mean, Standard Deviation, Minimum, Maximum) and Principal Components Analysis (PCA) were performed in the Past software (ver. 4.09; Hammer et al. 2001).

Some characters were not measured in each individual due to deformations caused by genital malformations and changes (mainly compound eye collapses) in older or incorrectly stored material.

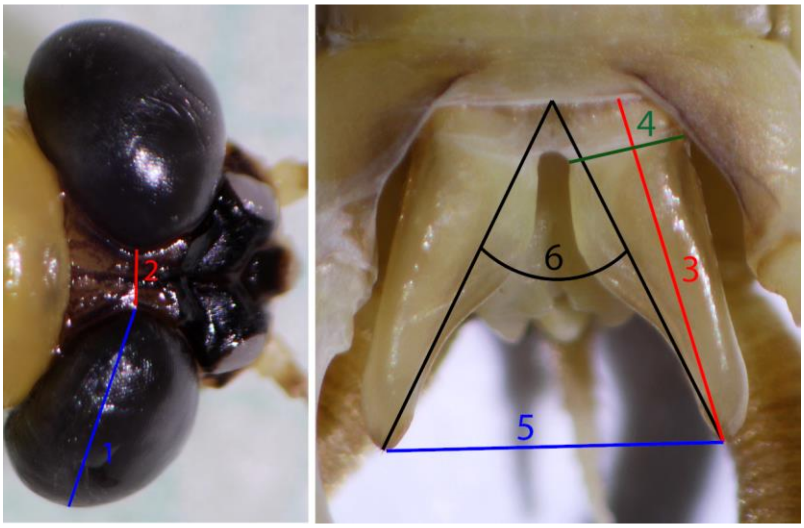

Fig.6 Measured characters on male specimens of *Palingenia* (1 – size of compound eye, 2 – distance between compound eyes, 3 – penis lobe length, 4 – penis base width, 5 – distance between penis lobe tips, 6 – penis lobe angle)

2.3 Molecular methods and data analyses

Tissue samples from 27 individuals were prepared and delivered according to the prescribed standards to the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph), where DNA barcodes were obtained using the standard high-throughput protocol described in deWaard et al. (2008). Specimen collection data, photographs, sequences, PCR, sequencing primers and trace files are available through the Barcode of Life Data Systems (BOLD; Sujeevan & Hebert 2007) under the project name Macro-zoobenthos from Romanian freshwaters [ROMAC]. Three other sequences were also generated at the CCDB through the Barcoding Diptera from the Romanian freshwaters project [RODI]. An additional 43 individuals were processed at the Interdisciplinary Research Institute on Bio–Nano–Sciences of Babeș–Bolyai University. Genomic DNA was extracted using a commercial kit (ISOLATE II Genomic DNA Kit, Bioline), and the *mt*COI sequences were amplified using the standard LCO1490 and HCO2198 primer pair (Folmer *et al.* 1994) in a 50 µl volume at 42°C. Sequencing was performed by Macrogen Inc. (Europe).

The number of haplotypes and polymorphic sites (S), the haplotype (Hd) and nucleotide diversity (π) were calculated in DnaSp 6 (Rozas *et al.* 2017). A haplotype network was built by implementing the Median-Joining (MJ) algorithm in PopArt 1.7 (Leigh & Bryant 2015). Individuals were coloured on the network based on the rivers they were collected on, in order to visualize the geographic distribution of the haplotypes. The *p*-distance between haplotypes and between populations of different rivers was calculated in Mega X (Kumar *et al*. 2018).

2. Results

3.1 Morphometry results

The results of the morphological analyses point to a relatively large variability between populations, with significant differences between sample medians of different populations in all analysed morphological characters (Tab. 3). However, the differences were also large within the populations, often showing double or triple ratio values. This was observed, for example, in the ratio between length and distance of the penis lobes between several populations (Danube – Bulgaria, the Danube Delta – Romania, the Dniester, the Horyn – Ukraine, the Latorica, the Tisa – Slovakia, the Mureș – Romania); in the angle between the penis lobes (the Danube Delta – Romania, th Dniester, the Horyn – Ukraine, the Latorica, the Tisa – Slovakia, the Mureș – Romania); or the ratio between distance and width of compound eyes (the Horyn – Ukraine) (Tab.4).

The analysis of the main components used to explore the structure of the variation based on the sets of characters did not show any separation of the populations or species (Fig.7).

Tab 3. Results of Kruskal-Wallis test of equality of medians

Kruskal-Wallis	**L-D-ratio**	**Ratio-lobes**	**Angle-lobes**	**Ratio-eyes**
H (chi2)	48.03	42,00	59.28	62.68
p (same)	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Tab 4. Results of the univariate statistics of particular morphological characters of individuals collected in different sampling locations/different populations

Population	**Danube-BG**	**Danube-Del-C-RO**	**Danube-Del-RO**	**Danube-HU**	**Dnister-UA**	**Horyn-UA**	**Latorica-SK**	**Latorica-SK1**	**Mureș-RO**	**Prut-RO**	**Tisa-SK**
Ratio between length and distance of penis lobes											
N	12	4	22	2	2	22	24	17	25	30	14
Median	1.009	1.159	1.154	1.136	1.747	1.099	1.079	1.255	1.428	0.997	1.251
Mean	1.070	1.155	1.250	1.136	1.747	1.105	1.153	1.302	1.506	1.006	1.462
Std. Deviation	0.212	0.069	0.401	0.008	1.053	0.147	0.258	0.288	0.438	0.092	0.506
Minimum	0.829	1.071	0.812	1.130	1.002	0.895	0.950	0.962	0.944	0.851	1.043
Maximum	1.597	1.231	2.135	1.141	2.491	1.625	2.179	2.241	3.009	1.232	2.907
Ratio between the length and width of penis lobes											
N	12	4	22	2	2	22	24	17	25	30	14
Median	2.705	2.588	2.530	2.811	1.997	2.466	2.518	2.814	2.538	2.591	2.655
Mean	2.629	2.537	2.494	2.811	1.997	2.439	2.485	2.788	2.521	2.587	2.728
Std. Deviation	0.262	0.142	0.220	0.169	0.355	0.119	0.165	0.171	0.208	0.193	0.261
Minimum	2.014	2.333	1.998	2.691	1.746	2.191	2.124	2.423	2.071	2.283	2.389
Maximum	2.893	2.639	3.032	2.931	2.248	2.641	2.817	3.021	2.851	3.000	3.213
Angle between penis lobes											
N	12	4	22	2	2	22	24	17	25	30	14

Median	56.471	47.746	47.918	51.691	32.661	50.847	50.695	50.943	40.218	56.554	51.035
Mean	56.411	47.832	48.794	51.691	32.661	50.641	49.959	50.031	40.912	55.545	45.568
Std. Deviation	8.543	4.079	13.609	0.022	16.851	6.573	7.805	8.616	10.493	5.121	11.093
Minimum	38.356	43.289	26.463	51.675	20.746	32.847	26.107	26.633	17.804	45.493	19.225
Maximum	67.216	52.546	68.633	51.706	44.577	64.279	65.175	62.127	64.123	64.539	58.208
						Ratio between distance and width of compound eyes					
N	11	4	22	2	2	23	24	20	8	30	7
Median	5.130	3.881	4.442	3.778	5.594	4.397	4.044	3.905	5.831	5.135	4.238
Mean	5.476	4.004	4.466	3.778	5.594	4.558	4.082	3.945	5.625	5.115	4.467
Std. Deviation	0.841	0.667	0.540	0.145	0.421	0.834	0.465	0.540	1.039	0.692	0.600
Minimum	4.606	3.408	3.407	3.675	5.297	3.331	3.148	3.178	4.121	3.659	3.582
Maximum	7.108	4.847	5.216	3.881	5.892	6.869	5.107	5.329	7.026	6.632	5.208

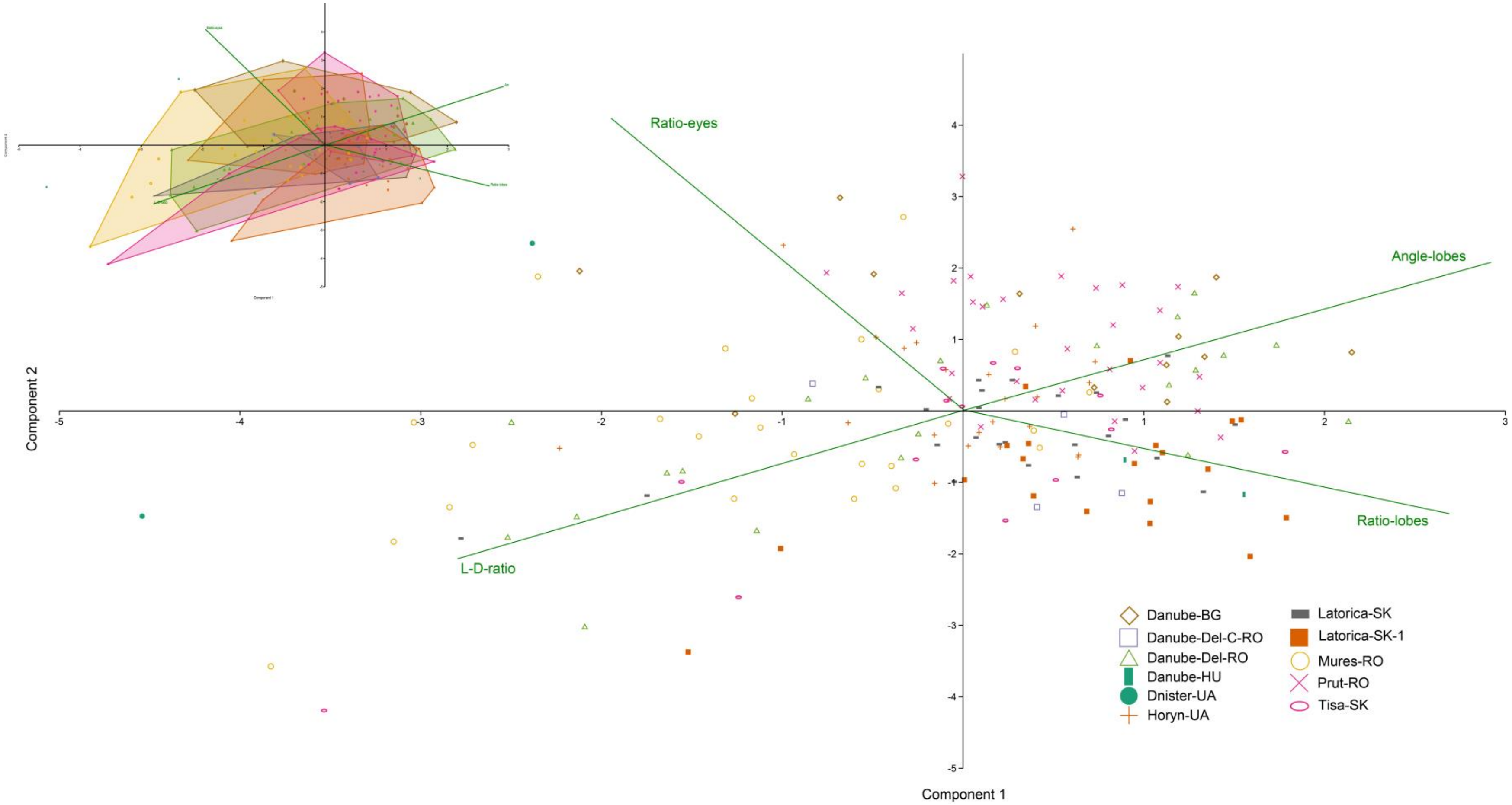

Fig 7. Principal component analysis (PCA) biplot for morphometric characters (the percentage of total variance associated with PC1: 68.82%; PC2: 22.00%). Different colors and symbols represent different sampling locations. In the upper left corner, an identical graph is shown with the marking of polygons corresponding to different populations to show the overlap.

3.2 Molecular results

The 73 sequences showed 15 polymorphic sites leading to 15 haplotypes, with a haplotype diversity of Hd= 0.576 and a nucleotide diversity of π= 0.00252. Two haplotypes were common, corresponding to 46 and 13 individuals, and one haplotype was shared by two specimens collected at the same location. The other 12 haplotypes were unique, represented by only one individual (Fig.8) The *p*-distance between haplotypes ranged between 0.159 and 1.297. When sequences were grouped based on different rivers, the *p*- distance showed values between 0.22 and 0.89 (Tab.5).

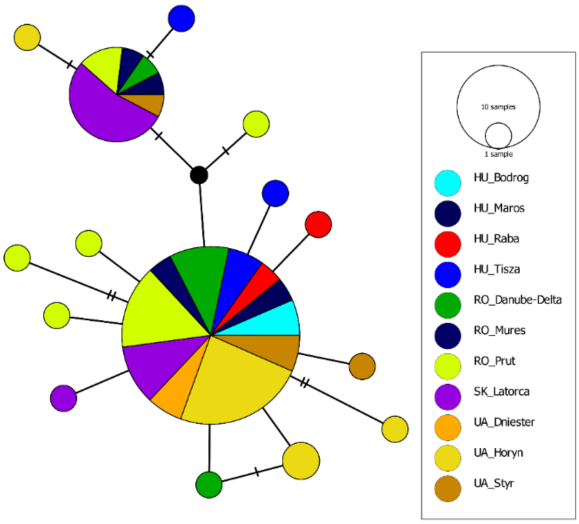

Fig.8 Median-Joining haplotype network generated for the concatenated dataset using PopArt 1.7. Each circle represents a unique haplotype and circle size is proportional to the number of samples observed for that haplotype. The number of mutations is represented by hatch marks on the lines. Colors correspond to different rivers.

Tab. 5 *p*-distance (%) between populations of different rivers.

	1.	**2.**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1. RO_Prut										
2. Hu_Maros	0.43									
3. Hu_Bodrog	0.65	0.60								
4. HU_Raba	0.38	0.39	0.62							
5. HU_Tisa	0.57	0.51	0.59	0.54						
6. UA_Horyn	0.41	0.41	0.61	0.31	0.54					
7. UA_Dniester	0.31	0.34	0.45	0.30	0.44	0.27				
8. SK_Latorca	0.56	0.46	0.89	0.49	0.66	0.56	0.53			
9. RO_Danube Delta	040	0.38	0.63	0.25	0.53	0.35	0.29	0.50		
10. RO_Mureș	0.44	0.39	0.72	0.37	0.56	0.42	0.36	0.46	0.39	
11. UA_Styr	0.42	0.38	0.72	0.22	0.56	0.36	0.34	0.45	0.32	0.38

3. Discussion

Based on our morphometry results of the penis morphology and eye distances on males, no significant differences support the clear separation of the analyzed populations and evidence of more than one *Palingenia* species (*P. longicauda*) in Europe.

Redescription of the European species belonging to the genus *Palingenia* (Soldán, 1978) was most probably based on the incorrect assumption of the occurrence of *P. fuliginosa* in Slovakia. Features listed by the author as distinctive in species identification were reanalysed and proven to be based on the intraspecific variability observed in this study (Fig.9). Moreover, it is also important to note that some features are greatly influenced by the time at which individuals were fixed in ethanol after the sub-imagos had moulted. This applies particularly, for example, to the angle between the lobes of the penis. In the case of a sub-imagos, the angle between the lobes is very sharp, and the penis lobes are essentially parallel. After moulting, the angle increases until it stabilizes. If individuals are collected and fixed in the pre-stabilization period, the angle varies considerably. Thus, we consider the morphological differences mentioned by Soldán (1978) between the males of the two species, *P. longicauda* and *P. fuliginosa,* highly challenged and extremely variable, even within the same population, depending mostly on moulting status of the examined individuals.

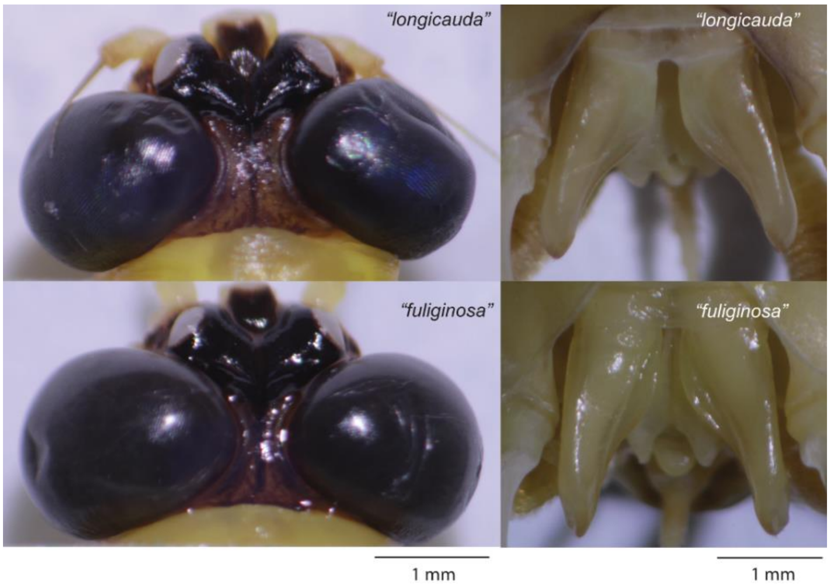

Fig. 9 Differences in male genitalia structures and dimensions of compound eyes in individuals with highly similar mtCOI structures and sampled at the same time from a single population (Latorica, Slovakia). The specimens depicted here are morphologically close to the description and characters shown in Soldán's revision as *P. longicauda* and *P. fuliginosa* (designated "*longicauda*" and "*fuliginosa*", respectively).

Mitochondrial DNA analysis showed similar patterns to those observed in the previous molecular genetic studies of *P. longicauda* (Bálint *et al.* 2012; Dénes *et al.* 2022), with a low number of haplotypes present in the whole distribution area and several other private haplotypes present in different rivers. The MJ haplotype network did not show any evident or well-differentiated structures to indicate that the sequences could represent more than one taxonomic unit. The low *p*-distances, both when the haplotypes were compared (0.159 – 1.297) and when the different river populations were compared (0.22 – 0.89) are consistent with previously published intraspecific distance values for the *Palingeniidae* species (Webb *et al.* 2012: 1.6% maximum intraspecific and 12.7% minimum interspecific distances). These results support the micromorphology data, thus confirming the presence of a single *Palingenia* species, *P. longicauda,* in the south-eastern part of Europe.

According to Soldán (1978), and consequently adopted by an important number of authors following (Andrikovics & Turcsányi 2001; Martynov 2018), the major argument for the presence of *P. fuliginosa* in Europe is the species' control for different ecological demands compared to *P. longicauda.* Their nymphs were frequently collected in smaller rivers with unpolluted and rapidly flowing waters and high oxygen supply, while *P. longicauda* is present only at lower sectors of large rivers with a lower oxygen content. However, there were also some contributions that noticed the presence of the two morphotypes of *Palingenia* in a same river sector (ex. Bodrog in Hungary; Málnás et al. 2016) or even different species in the same sample (Bodrog in Slovakia) (Mišíková Elexová *et al.* 2015), which makes the above-mentioned argument questionable, and the presence of *P. fuliginosa* in the hydrographic basin of the middle sector of the Tisza river highly doubtful. In contrast, these findings refute Soldán's argument and indirectly support the presence of only a single species, *P. longicauda,* in the south-eastern part of Europe.

The taxonomic status of *P. fuliginosa* remains challenged, however, as the type material is missing, or its location is unknown, and our repeated efforts to obtain fresh material from outside of Europe (from Iran, Armenia, Azerbaijan, for example) have failed. Until a better taxonomic solution is found, we suggest reinstalling *P. fuliginosa* as a junior synonym of *P. longicauda.*

The situation of the third European species, *P. sublongicauda*, is similar, as no adult male material were available to us prior to morphological investigation and no type material was available during the present study.

The major contribution of the present paper is the first integrative analysis of morphology and molecular data of the *Palingenia* species recorded from the south-eastern Europe. Besides our strong argument on the presence of only one, instead of three *Palingenia* species in Europe, further comprehensive sampling efforts are highly recommended, including an analysis of all species from the Palaearctic area, but also a revision on Indo-Malayan and Afrotropical representatives, thus a well-supported taxonomic revision of the genus.

Our study also has important conservation implications, as all Palingenia species have high bio-indication value of the ecological integrity of medium-sized to large pristine rivers and are critically endangered in large part of the known distribution area (Russev 1987; Soldán et al. 2009; Bauernfeind & Soldán 2012).

Chapter IV

The social impact on the conservation of the species *Palingenia longicauda* (OLIVIER,1791) in Romania

Identification of cryptic populations based on methodology "*citizien science*"

The real natural phenomenon of mass swarming of adults of Palingenia longicauda was of great social interest in the past, since it is already known from the literature that this species was widely used as fishing bait. It was popularly known under various names: *"oeveraas"* and *"haft"* în Netherlands, *"Spork-Oese"*, *"Sprock"*, *"Spaargoos"*, *"Spaargaanse"* in Germany, *"Tiszavirag"* in Hungary, *"gandatsi"* for larvae and *"rusalki"* or *"karchani"* for adults in Bulgary (Russev, 1987). In Romania it is popularly known under the name 'flower of the rivers' on the Crișuri rivers, '*rusalii'* in the Danube Delta and the Mureș river, and on the Prut river, under the name 'vetrică'. In the current network of digitized information, including various social media platforms, a lot of information about this species appears, either as a tourist attraction or as important information among fishermen; they are uncertain data, but which document an unexpected recovery of the species in a remote area, such as the Danube Delta, the Mureș or the Prut river in Romania.

Due to the fact that the information on social networks has multiplied, we have decided to turn to these '*citizen scientists'* to capitalize on the data they observed, through a survey. The survey was carried out with the help of a questionnaire that was based on questions about the recognition of the P. longicauda species based on photographs (both the larva and the adult insect), fishing practices (collecting the insect and using it as bait), as well as questions regarding the location where the species is present, knowledge about the presence of the species before the year 2000, and the respondents' opinion related to the size of the observed populations. The questionnaire was applied to residents, nature enthusiasts and especially fishermen on the Prut, Mureș and Danube Delta rivers. A total of 160 people completed the questionnaire: 60 people on the Prut river, 50 on Mureș river and 50 in the Danube Delta.

The questionnaire was completed only by the people who recognized the species, the majority of whom had personally seen a swarm of the insect (Danube Delta - 76%; Mureș - 98%; Prut - 91.66% of respondents) at the end of May - beginning of June . Based on the information obtained, the presence of the species P. longicauda was confirmed in the lower part of the Danube, near the towns of Spantov, Borcea, Capidava and Galați, in the Măcin-Old Danube Branch, and in several locations in the Danube Delta. Similar results were also obtained on the Mureș river, where the species was reported on most of the section between Sâmbeteni and Nădlac and on the Prut river between Lunca Banului (the southernmost point reported) to the north of the country, at Rădăuți-Prut.

The answers regarding the presence of the species before the 2000s differ in the case of the three rivers studied. In the Danube Delta, more than half of the respondents (55.1%) answered that they had observed the presence of the species before 2000, on the Prut River approximately a quarter (26.67%) confirmed the presence of the species, and on the Mureș River almost two thirds (68%) (Fig. 10). The numbers of the species are currently seen as increasing in the Danube Delta by 44% of the respondents, and 26% consider that the number of individuals is stable. On the Prut and Mureș rivers, about half of those asked consider the population size to be stable, and an equal number of people see this number as increasing or decreasing (Fig. 10).

Fig. 10. Responses regarding the presence of the species before the 2000s (left). Answers regarding current numbers of the species (right)

These observations are in accordance with the results obtained on the basis of the population genetics study. The species has been considered extinct in these rivers because population sizes have declined drastically following pollution and hydro-morphological interventions, making the species harder to detect, with small flocks lacking spectacular swarmings.

In Romania, as well as at European and global level, this species is not evaluated, and based on our results we proposed changing the IUCN status of this species from Not Evaluated in Romania to Least Concern. In order to achieve this objective, the main step was the reassessment of the species based on the guide proposed by the International Union for Conservation of Nature (IUCN), which is done by the members of the Red List Authorities based on the existing scientific data.

The identification of new distribution areas of the species Palingenia longicauda and the reporting of viable populations in these areas, show a low degree of endangerment from the point of view of the IUCN Red List, and thanks to the effort made in this research, we have provided all the necessary data for the reevaluation of the species based on the guide proposed by the International Union for Conservation of Nature (IUCN), so that P. Longicauda is now included in the category of non-endangered species (Least Concerning - LC) (Macadam, C., 2023).

FINAL CONCLUSIONS

The results obtained as a result of the research activity carried out as part of the doctoral thesis, are in accordance with the proposed objectives:

- We have identified, collected and processed using population genetics methods several populations of Romania, and based on a large sampling effort of the *P. longicauda* species in its currently known range, in South-Eastern Europe and through collaboration with researchers from Hungary, Ukraine and Slovakia, we analysed several populations, and compared with additional specimens, including *P. fuliginosa* and probably *P. sublongicauda* from Landa and Soldán's collection, thus confirming the presence of *P. longicauda* with viable populations in southeastern Europe.
- The genetic differences based on the studied mitochondrial sequences show a significant differentiation between the populations of the Tisza river basin and the Rába river compared to the populations of the Danube Delta and Prut, suggesting that this species had at least a refuge in the Pontic area.
- The observed genetic differentiation confirms the fact that the populations rediscovered after the 2000s in Romania are autochthonous populations that survived through small populations in the Danube Delta and the Prut, and are not the result of a recent migration from the Tisa basin.
- We performed the first integrative analysis of morphological and molecular data of *Palingenia* species recorded from South-Eastern Europe. Our morphometric and molecular genetic results show the presence of only one species of *Palingenia* in the studied area, so the flagging of the *P. fuliginosa* species from Ukraine and Slovakia is the result of the misidentification of the high variability represented by the individuals of the *P. longicauda* species.

- The questionnaires carried out confirm the continuous presence of the species in the Danube Delta and on the Mureș and the Prut rivers, showing that it is widespread in the lower and middle courses of the studied rivers.
- The identification of new distribution areas of the species *Palingenia longicauda* and the reporting of viable populations in these areas, show a low degree of endangerment from the point of view of the IUCN Red List, and thanks to the efforts made in this research, we have provided all the necessary data for the reevaluation of the species based on the guide proposed by the International Union for Conservation of Nature (IUCN), so that P. *longicauda* is now included in the category of non-endangered species (Least Concerning - LC).

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Own publications

1. Manko P., **Vaida R.M.** *, Keresztes L., Martynov A., Szabó E., Baranová B., Kis B., Váncsa E, Dénes A.L. 2023, *Integrative taxonomy supports one rather than several species of Palingenia in South-Eastern Europe (Insecta, Ephemeroptera, Palingeniidae)*- The European Zoological Journal, 296-306, Vol.90, No.1; https://doi.org/10.1080/24750263.2023.2191622;
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