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DNA barcode data for the Carpathian headwaters: species-level identification of stonefly (Insecta, Plecoptera) larvae in a biodiversity hotspot of the Apuseni Mountains

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Abstract. Stoneflies are important ecological indicators of aquatic ecosystems, yet poorly explored due to their difficult species-level taxonomy and cryptic larvae forms. Nowadays, barcoding is widely used in biodiversity assessments, complementing morphological identification and requiring comprehensive reference DNA sequence databases. The DNA barcode region exhibits high interspecific and low intraspecific variation, making it a suitable tool for species delimitation, detecting cryptic biodiversity and species-level identification of morphologically cryptic life stages. In Romania, there are 132 (of which 24 are dubious) known species of stoneflies, of which 74 (57%) have standard barcode sequences in databases generated by European countries. Here we present a DNA barcode initiative based on local populations of Plecoptera, which aims to create molecular data of stoneflies from a well-known aquatic biodiversity hotspot in the Romanian Carpathians, the Apuseni Mountains. 175 specimens were collected between 2020 and 2021 and analyzed using the standard barcode region (*mt*COI). The 97 larvae and 62 adult sequences represented 29 known species, of which six lacked a Barcode Index Number (BIN). We further identified six previously unknown new BINs, indicating overlooked diversity or providing new molecular data for existing taxa, and suggesting the presence of cryptic or undescribed species. In total, we added 12 new BINs to the Barcode of Life Data System. We managed to associate 86 larval stage Plecoptera specimens with 24 known species using barcode sequences. For the first time, we generated DNA barcode data for one endemic and two sub-endemic species of the Carpathians: *Brachyptera starmachi* Sowa, 1966, *Leuctra carpathica* Kis, 1966, and *Protonemura aestiva* Kis, 1965. Our results provide a more realistic estimate of the aquatic biodiversity in the Apuseni Mountains and underline the importance of local datasets to support regional aspects of the larvae-taxonomy for Plecoptera from Romania.

Keywords: cryptic diversity, *mt*COI, aquatic insects, endemism, BIN numbers, larvae.

Introduction

Freshwater ecosystems support high biodiversity, covering approximately 10% of all known species, and are an essential natural resource for the growing human population through their complex ecosystem services, including their diverse biodiversity and quality (Strayer & Dudgeon 2010, Macadam & Stockan 2015). Despite this, freshwater habitats are degrading at a high rate globally and their fauna are at constant risk of extinction, even before we can fully explore their biological complexity (Sayer et al. 2025).

The aquatic habitats of mountainous regions, with their cryptic diversity, unique evolutionary histories, and significant regional specificity, are more endangered than all other natural resources (Albert et al. 2021). Their diversity is rapidly decreasing due to the increasing anthropogenic pressure and global climate change (Bálint et al. 2011a). Therefore, research aimed at assessing ecological integrity, through species-level identification, can provide environmental managers and decision-makers to take appropriate and effective actions to the long-term management of these valuable natural resources (Leese et al. 2016).

Aquatic insects are important components of freshwater biota by providing unique ecosystem services in nutrient cycling and energy flow (Macadam & Stockan 2015). Despite this, the species-level identification of aquatic insects, mostly larvae, based on classic morphological criteria is still difficult to achieve, mainly due to the lack of morphological characters suitable for reliable data (Jackson et al. 2014).

Aquatic ecosystems in Europe are well-known biodiversity hotspots for several range-restricted, endemic insect species, inhabiting mostly fast-flowing mountain streams, and have been intensively explored through case studies in many species-complexes in the case of Ephemeroptera (Leys et al. 2016, Vuataz et al. 2016), Plecoptera (Gattolliat et al. 2016, Vitecek et al. 2017a), Trichoptera (Previšić et al. 2014, Graf et al. 2015, Vitecek et al. 2017b) and Diptera (Ujvárosi & Bálint 2012, Dénes et al. 2016).

The Carpathians are a major part of the European Alpine system, with important hotspots of biodiversity (Mraz & Ronikier 2016) and a significant number of rare, endangered, or endemic species linked to aquatic environments (Ujvárosi & Bálint 2012, Pârvulescu et al. 2020, Macko et al. 2023), but still underexplored in case of morphologically difficult taxa, like Ephemeroptera (Macko et al. 2024), Plecoptera, Trichoptera (Bálint et al. 2011b), Diptera (Dénes et al. 2016), and Amphipoda (Copilaş-Ciocianu & Petrusek 2015, Copilaş-Ciocianu et al. 2018a). Among the already recognized biodiversity hotspots from here, the Apuseni Mountains is one of the most important biogeography regions of the Carpathians, due to real insularity during the Miocene, and

lack of glaciated surfaces in the Pleistocene Ice ages (Pop 1997, Pop et al. 2010, Mraz & Ronikier 2016, Trájer 2023). Thus, this mountainous region deserves special attention, because of its complex geomorphological and ecological history, which resulted in a considerable number of endemic species and highly divergent genetic structures (Mey & Botosaneanu 1985, Bálint et al. 2011a, Pârvulescu et al. 2013, 2020, Copilaş-Ciocianu & Petrusek 2015, Antal et al. 2016, Dénes et al. 2016).

DNA-based technologies have the potential to act as "game-changers" in freshwater ecosystem assessments. Methods, like DNA barcoding, are a useful tool for delimitating and identifying species in most cases, as the 658 bp mitochondrial cytochrome c oxidase subunit I (COI) fragment, has low intraspecific variation and high interspecific divergence in a large number of invertebrate taxa (Pentinsaari et al. 2014). Thus, DNA barcode data assure a strong basis for species-level identification of aquatic insects in all life stages, including morphologically cryptic forms and life stages (Zhou et al. 2010, Cardoni et al. 2015, Gattolliat et al. 2015, Copilaş-Ciocianu et al. 2022, Fahldieck et al. 2024)

Stoneflies (Plecoptera) are among the most important bioindicator aquatic insect groups, because of their sensitivity to organic pollution, specialist feeding type, endemism at high altitudes, and the preference for cold, fast-flowing and highly oxygenated waters as larvae (Souza et al. 2024). However, the species-level larvae identification is challenging, limiting their application in freshwater biodiversity assessments. Applying DNA barcoding to associate cryptic larvae with adult specimens based on genetic sequences can improve assessments and solve this limitation (Molina et al. 2017). To this end, comprehensive and reliable DNA barcode reference libraries have been created for several European countries in the past decade (Gattolliat et al. 2016, Morinière et al. 2017, Ferreira et al. 2020, Hlebec et al. 2022, Laini et al. 2024, Vuataz et al. 2024). For Romania, however, the DNA sequences of this group are scarce and practically non-existent in any of the international databases (BOLD, NCBI).

The known species number of Romanian stoneflies is a bit obscure. Kis (1974) reports 122 species, among them the occurrence of 21 species, noted as unsure or considered erroneous in subsequent publications. Additional species were reported or described from Romania in the following papers: Kis 1972 (*Brachyptera bulgarica*), Murányi 2006 (informal records of four new species, three of them still undescribed), Vinçon & Murányi 2007 (*Leuctra dalmoni*), Murányi 2008 (*Taeniopteryx araneoides*), Vinçon & Murányi 2009 (*Rhabdiopteryx harperi*), Murányi et al. 2020 (*Isoperla nagyi*), Dénes et al. 2021 (*Zwicknia acuta*) and Murányi et al. 2023 (*Nemoura kozari*). Taking all these into account, there are 108 species of stoneflies in Romania with confirmed records, further 3 undescribed and 21 with dubious records, furthermore *mt*COI data are still lacking for local populations. 74 species belonging to the country's fauna have barcode sequences in some global databases (BOLD, GenBank) but none of them refer to the Romanian populations. Thus, our main objective was to initiate for the first time a comprehensive DNA Barcode library of Plecoptera from Romania, focusing on an important aquatic biodiversity hotspot in the Carpathians, the Apuseni Mountains. We aimed to test the usefulness of *mt*COI data for the biodiversity study of the local stonefly fauna from here, including morphologically cryptic larvae. Finally, we propose to answer the question: Is there room for additional DNA barcode data to support a high-quality bio-assessment of stoneflies from here, complementary to the existing public DNA barcode data?

Materials and methods

Study area and sampling sites

The study area includes headwaters of the Someşul Cald River on the perimeter of the Apuseni Natural Park Protected Area. The sampling sites include rheocrene springs and first- and second-order brooks above 1000 m, which are well-preserved, diverse, natural freshwater habitats. A total number of 31 different sites were investigated in 2020 and 2021 (Fig. 1). Larvae specimens were selected from macrozoobenthos samples, while adult specimens were collected individually with entomological nets along the different water bodies.

Morphological identification

Adults and larvae were selected from a bulk sample containing 3,052 individuals. We analysed 175 specimens representing 6 families and 14 genera and classified them into 36 morpho-species (Table 1.). Classification and scientific names follow the Global Biodiversity Information Facility (https://www.gbif.org). Adult males and females were identified to species-level by using Kis (1974). Larvae were identified to the most appropriate taxonomic level that we could reach relying on morphology, by using available literature data (Aubert 1946, Raušer 1956, Zwick 2004, Murányi & Kovács 2015).

Individuals of adults and larvae were collected in large collection tubes containing more specimens in 96% ethanol in the field. Afterwards, in the laboratory, we selected these samples, and specimens were identified, coded, and separately preserved in 96% ethanol. They were stored at -20 °C until tissue sampling and deposited as vouchers in the Macro Invertebrates Collection (MIC-RO) of the Faculty of Biology and Geology, Babeş-Bolyai University in Cluj-Napoca (BBU), Romania.

Barcoding and data interpretation

DNA was extracted from 175 specimens. Legs or body fragments to 2 mm of selected specimens were placed in 96-well microplates and sent to the Canadian Centre for DNA Barcoding (CCDB) for DNA extraction, PCR, and sequencing of the 658 bp *mt*COI barcode region using the CCDB standard high-throughput protocols (available at: http://ccdb.ca/resources). Details of PCR and sequencing primers for all samples, the barcode sequences, and the trace files for these sequences were uploaded to the Barcode of Life Data (BOLD) Systems database, under ROMAC project name for storage and analyses, along with all relevant collection data and photographs of the specimens. Taxa names were introduced into the BOLD system based on our previous rigorous morphological examination on voucher specimens.

Data were analyzed based on information present on the BOLD platform on 30.01.2025. Every analysis was made using sequences with 500 or more base pairs (bps) which fulfilled the barcode compliance criteria (Pentinsaari et al. 2014).

Barcode Index Numbers (BINs) appear as species proxies that are proposed to validate the taxonomic units for sequences grouping together, through a multi-step algorithm called Refined Single Linkage (RESL) Analysis (Ratnasingham & Hebert 2013). This newly proposed methodology uses morphology-based taxonomic grouping to recalculate the genetic thresholds with every newly added sequence in the genetic database.

The "BIN Discordance" tool on BOLD was used to analyze the concordance between BINs and species designations. Intraspecific, congeneric (between species within a genus) and confamiliar (within families) distances were estimated for the whole dataset based on *p*-distance using the tool (Distance Summary) available on the BOLD

platform.

The barcode gap between the maximum intraspecific genetic distance and minimum interspecific distance (Ratnasingham & Hebert 2013) was tested with the "Barcode Gap Analysis" tool available on the BOLD platform. Species presenting a maximum intraspecific distance value lower than the minimum interspecific distance were considered successfully discriminated.

The number of haplotypes and polymorphic sites (S), the haplotype (Hd), and the nucleotide diversity (π) were calculated in DnaSp 6 (Rozas et al. 2017). A Bayesian Inference phylogenetic tree was implemented in MrBayes (Ronquist et al. 2012), with a GTR+G+I model. Two mayfly (Ephemeroptera) sequences, namely ROMAC105 — *Epeorus assimilis* Eaton, 1865 (BIN: AAF2291) and ROMAC160 — *Ecdyonurus carpathicus* Sowa, 1973 (BIN: AEC7894) were used as outgroups for the three constructions. The tree was visualized using FigTree ver.1.4.3 (Rambaut 2009) and iTOL ver. 5 (Letunic & Bork 2021).

The species-level identification of stonefly larvae was based on the genetic similarity between larvae barcodes and adult sequences in the BOLD system, generated either by the present study or compared with sequences already uploaded to some open-access genetic databases (NCBI, BOLD).

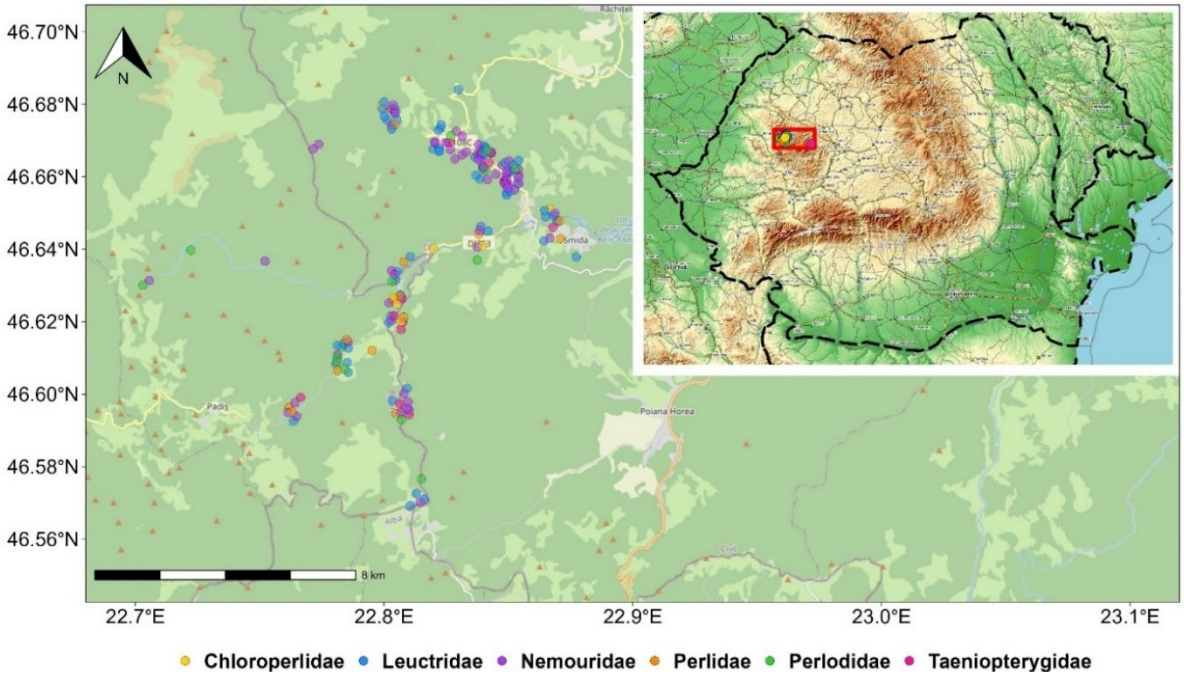

Figure 1. The location of the Apuseni Mountains, Romania and the detailed map of the collection sites, at the headwaters of Someşul Cald River. Each coloured dot represents a collected specimen, while the colours refer to the stonefly family it belongs to. Due to the fact that multiple points were recorded in relatively small areas, a slight jitter effect was applied to the points on the map to enhance visibility.

Results

Morphology

The identification of adult specimens relying on morphology resulted in 26 adult male specimens belonging to 16 known stonefly species, and 29 adult female specimens representing 15 species (Table 1). In the case of adult males, 3 specimens were associated only with the genus *Isoperla* Banks, 1906 due to bad conditions of male genitalia. After barcode sequences were obtained, they proved to group in 2 new BINs (*Isoperla sp. 1 RO* - BIN: AET0817 and *Isoperla sp. 2 RO* - BIN: AER8567). Among adult female specimens, there were four cases when morphological identification reached just the genus level (3 *Isoperla* specimens and 1 *Leuctra* specimen), later on, barcode sequences revealed that both of them belong to previously unassigned BINs of the genera (*Isoperla sp. 1 RO* - BIN: AET0817 and *Leuctra sp. 1 RO* BIN: AES3405). Of all adult specimens, only 2 females remained unidentified (2 females from the genus *Protonemura* Kempny, 1898) due to the lack of distinct morphological characters and the failure of the sequencing process.

The species-level identification of the 111 stonefly larvae, based solely on morphology, resulted in 35 specimens associated correctly with six species, the rest of the specimens (76), were identified only to genus level or proved misidentified. Of the 111 larvae, 14 specimens remained identified just to genus level, because no barcode sequences were obtained successfully. The species-level identification success based on morphology data only has a ratio of 31.53% from the total material we analyzed (Table 1.).

Sequencing and genetic distance analyses

Out of the 175 specimens, sequencing was successful for 159 individuals, resulting in good-quality sequences longer than 500 bp in length, with a sequencing success rate of 90.86%. Sequences showed high adenosine and thymine (AT)- rich bias (average 60.84%) in concordance with the data from the literature (Ge et al. 2021). The 159 sequences represent 99 haplotypes based on S = 169 variable sites, with a haplotype diversity of Hd = 0.988 and a nucleotide diversity of π = 0.161.

The 159 sequences correspond to 62 adult and 97 larvae specimens, representing 27 concordant BINs and 9 singleton BINs, with no discordance between the morphologically identified species and the assigned BINs (Table 2.). BINs were considered "Known" if other sequences, besides those generated by our work, were assigned to them, or "New", if

only our sequences were grouped into them. Of the 36 BINs assigned to our dataset, 12 were new, representing a third of the investigated taxa. These showed distances to the nearest neighbors (NN)-BINs from the BOLD platform that show the lowest genetic distance values compared with the BINs from our dataset—ranging between 2.24% and 13.43% (Table 2.).

Out of the 12 new BINs (Table 2., marked with *) that we added to BOLD database, there were the first sequences of one endemic (*Leuctra carpathica* Kis, 1966 - BIN: AET7481) and two subendemic species (*Brachyptera starmachi* Sowa, 1966 - BIN: AEK3810 and *Protonemura aestiva* Kis, 1965 – BIN: AES1185), new BINs of regional lineages for four species (*Leuctra cf. prima 2 RO* – BIN: AEK2888, *Nemoura cambrica* Stephens, 1836 – BIN: AEL4026, *Nemoura mortoni* Ris, 1902 – BIN: AES5545 and *Siphonoperla neglecta* (Rostock, 1881) - BIN: ACB5466) and five new BINs representing up to present unknown taxa (*Leuctra sp. 1 RO* – BIN: AES3405, *Nemoura sp. 2 RO* – BIN: AEU5267, *Nemoura sp. 3 RO* – BIN: AEU5265, *Isoperla sp. 1 RO* – BIN: AET0817, *Isoperla sp. 2 RO* – BIN: AER8567).

Table 1. Species-level identification of stonefly larvae based on morphology (first column) and barcoding (third column), with the calculated species-level identification success – SLI success (%), and the number of specimens from adult stages and different sexes (adult males – Ad-M, adult females – Ad-F) of the total 36 stonefly morpho-species analysed in this study.

Identification based on morphology	**Nr. Larvae**	**Identification based on barcoding**	**Nr. Larvae**	**Nr. Ad-M**	**Nr. Ad-F**
Brachyptera seticornis	9	*Brachyptera seticornis*	9	1	2
Brachyptera sp.	1	*Brachyptera starmachi*	1	2	0
Taeniopteryx sp.	1	*Taeniopteryx auberti*	1	0	1
Leuctra sp.	36	*Leuctra albida*	0	0	3
		Leuctra carpathica	1	2	1
		Leuctra cf. prima 2 RO	3	1	0
		Leuctra digitata	0	3	2
		Leuctra inermis	8	0	1
		Leuctra moselyi	1	2	1
		Leuctra nigra	12	1	4
		Leuctra quadrimaculata	1	0	1
		Leuctra sp. 1 RO	3	0	1
Amphinemura sp.	4	*Amphinemura sulcicollis*	3	0	0
Nemoura cinerea	3	*Nemoura cambrica*	1	1	0
Nemoura mortoni	1	*Nemoura cinerea*	2	0	0
Nemoura sp.	5	*Nemoura mortoni*	3	0	0
		Nemoura sp. 2 RO	1	0	0
		Nemoura sp. 3 RO	1	0	0
		Nemoura uncinata	1	1	1
Nemurella pictetii	16	*Nemurella pictetii*	15	2	2
Protonemura intricata	1	*Protonemura aestiva*	6	2	2
Protonemura montana	2	*Protonemura hrabei*	0	4	4
Protonemura sp.	7	*Protonemura intricata*	1	0	0
		Protonemura montana	0	1	0
		Protonemura praecox	3	1	0
Perla marginata	2	*Perla marginata*	2	1	3
Perla pallida	7	*Perla sp. 1 RO*	4	0	0
Dinocras sp.	1	*Dinocras megacephala*	1	0	0
Arcynopteryx dichroa	3	*Arcynopteryx dichroa*	3	0	0
Perlodes sp.	2	*Perlodes intricatus gr. form 2-GV*	1	0	0
		Perlodes microcephalus	1	0	0
Isoperla sp.	3	*Isoperla oxylepis*	0	1	0
		Isoperla sp. 1 RO	2	2	3
		Isoperla sp. 2 RO	0	1	0
Chloroperla sp.	3	*Chloroperla tripunctata*	3	0	1
Siphonoperla sp.	4	*Siphonoperla neglecta*	3	0	0
		Not sequenced	14	0	2
SLI success (%)	**31.53%**	***SLI success (%)***	**77.47%**	**29**	**35**

Table 2. Intraspecific mean *p*-distance divergences, maximum pairwise distances, Barcode Index Number (BIN), nearest species, distance to nearest neighbour (NN) (distances in %) of stonefly morpho-species included in the study. The * is marking the new BINs we added to the database. Source: DNA Barcode data from BOLD, ROMAC project.

Species	Mean Intra-Sp	Max Intra-Sp	BIN	Nearest Species	Dist. NN
Brachyptera seticornis	0.91	2.14	BOLD:AAY5851	*Brachyptera sp.*	11.44
Brachyptera starmachi	0.31	0.46	BOLD:AEK3810*	*Brachyptera monilicornis*	9.13
Taeniopteryx auberti	0.33	0.33	BOLD:ACB1944	*Taeniopteryx auberti Taeniopteryx hubaulti*	5.75
Leuctra albida	0.41	0.62	BOLD:AEH5504	*Leuctra meridionalis*	1.12
Leuctra carpathica	0.71	1.43	BOLD:AET7481*	*Leuctra cf. prima 1 RO*	11.38
Leuctra cf. prima 2 RO	0.15	0.31	BOLD:AEK2888*	*Leuctra joani*	5.66
Leuctra digitata	0.37	0.61	BOLD:AAK8671	*Leuctra sp.*	4.17
Leuctra inermis	0.50	1.22	BOLD:ADS4550	*Leuctra inermis*	2.07
Leuctra moselyi	0.15	0.31	BOLD:AAK8666	*Leuctra sp.*	5.61
Leuctra nigra	0.59	1.61	BOLD:AAK8665	*Leuctra nigra*	8.49
Leuctra quadrimaculata	0.33	0.33	BOLD:ADH8093	*Leuctra metsovonica*	8.17
Leuctra sp. 1 RO	0.89	1.53	BOLD:AES3405*	*Leuctra pseudosignifera*	3.72
Amphinemura sulcicollis	0.21	0.31	BOLD:AAK1251	*Amphinemura guadarramensis*	1.62
Nemoura cambrica	0.31	0.31	BOLD:AEL4026*	*Nemoura cambrica*	5.93
Nemoura cinerea	0.17	0.17	BOLD:AAG9376	*Nemoura cinerea*	2.66
Nemoura mortoni	0.20	0.31	BOLD:AES5545*	*Nemoura mortoni*	2.56
Nemoura sp. 2 RO	0.00	0.00	BOLD:AEU5267*	*Nemoura fusca*	11.7
Nemoura sp. 3 RO	0.00	0.00	BOLD:AEU5265*	*Nemoura fusca*	13.43
Nemoura uncinata	0.61	0.92	BOLD:AEU5266	*Nemoura uncinata*	2.4
Nemurella pictetii	1.24	2.49	BOLD:AAF0555	*Nemurella pictetii*	1.99
Protonemura aestiva	0.26	0.76	BOLD:AES1185*	*Protonemura auberti*	2.24
Protonemura hrabei	0.50	1.22	BOLD:ADR0060	*Protonemura hrabei*	2.08
Protonemura intricata	0.00	0.00	BOLD:AAM9758	*Protonemura intricata*	5.61
Protonemura montana	0.00	0.00	BOLD:ADQ8628	*Protonemura algovia*	6.26
Protonemura praecox	0.23	0.46	BOLD:AAK9862	*Protonemura praecox*	1.28
Perla marginata	0.52	0.88	BOLD:AAL2357	*Perla grandis*	2.46
Perla sp. 1 RO	0.46	0.77	BOLD:AAE6437	*Perla pallida*	1.92
Dinocras megacephala	0.00	0.00	BOLD:AAL0965	*Dinocras cephalotes*	3.39
Arcynopteryx dichroa	0.20	0.31	BOLD:AAM5773	*Skwala compacta*	4.67
Perlodes intricatus gr. form 2-GV	0.00	0.00	BOLD:ACD2664	*Perlodes jurassicus*	4.17
Perlodes microcephalus	0.00	0.00	BOLD:AEH5507	*Perlodes sp.*	3.2
Isoperla oxylepis	0.00	0.00	BOLD:AAN2092	*Isoperla grammatica*	1.9
Isoperla sp. 1 RO	0.76	1.24	BOLD:AET0817*	*Isoperla grammatica*	2.46
Isoperla sp. 2 RO	0.20	0.20	BOLD:AER8567*	*Isoperla grammatica*	2.84
Chloroperla tripunctata	0.93	1.54	BOLD:ACD8465	*Chloroperla sp.*	5.44
Siphonoperla neglecta	0.50	0.82	BOLD:ACB5466*	*Siphonoperla neglecta*	2.29

The *p*-distance analysis showed a clear gap between the maximum intraspecific distances, ranging from 0% to 2.49% (with an average 0.77%), and the minimum congeneric interspecific *p*-distances, with values between 4.42% and 17.76% (mean distance of 13.28%) (Fig. 2.). Although, the sequences of the Romanian populations show well defined taxonomic units, there were 13 cases where the *p*-distance to the NN is lower than the maximum intraspecific distance observed within our data. These values ranged between 1.12% (among *Leuctra albida* Kempny, 1899 – BIN: AEH5504 and *Leuctra meridionalis* Aubert, 1951 – BIN: AED2906) and 2.46% (among *Perla marginata* (Panzer, 1799) – BIN: AAL2357 and *Perla grandis* Rambur, 1842 – BIN: AEG8257, and also among *Isoperla sp. 1 RO* – BIN: AET0817 and *Isoperla grammatica* (Poda, 1761) – BIN: ADS2063) and represented distances among two morphospecies, or two BINs representing the same morphospecies.

All species/taxonomic groups from our dataset, predefined based on morphological identification, are grouped together in monophyletic clades with strong support on the BI tree (Fig. 3). Phylogenetic relationships are in concordance with the morphology-based hypothesis at the species and genus levels, but our dataset lacks sufficient phylogenetic signal to resolve relationships at higher taxonomic levels.

Using DNA barcode data to support species-level identification of unknown larvae

An integrative approach was used in this study for larvae identification. Of the 97 analyzed larvae, 65 were assigned to 15 species, based on adult individuals and larvae sequence associations (Fig.3). Additionally, 5 larvae corresponded to

two undetermined taxonomic units, designated as *Leuctra sp. 1 RO* (BIN: AES3405) and *Isoperla sp. 1 RO* (BIN: AET0817), which based on the adult specimen's morphology are likely existent species lacking molecular data. In the case of 3 larvae, we have found a new cryptic lineage of *Leuctra prima* Kempny, 1899, based on the morphology of the adult male specimen, that received a new BIN: AEK2888 and was designated *Leuctra cf. prima 2 RO*.

When lacking corresponding adult sequences in the material we collected, species-level identification was exclusively based on the genetic similarity of larvae barcodes and existing sequences in the BOLD database. Based on this approach, 18 larvae were further assigned to 9 species. Of the remaining 6 larvae, 2 represented two undetermined taxa with new BINs assigned (designated as *Nemoura sp. 2 RO* – BIN: AEU5267 and *Nemoura sp. 3 RO* – BIN: AEU5265), and the rest of them (4), belong to one known BIN: AAE6437, also an undetermined species, designated *Perla sp. 1 RO*. These results show an 88.65 % success rate of species-level identification for larvae that were successfully sequenced, and 77.47% for all the sampled larvae (Table 2.).

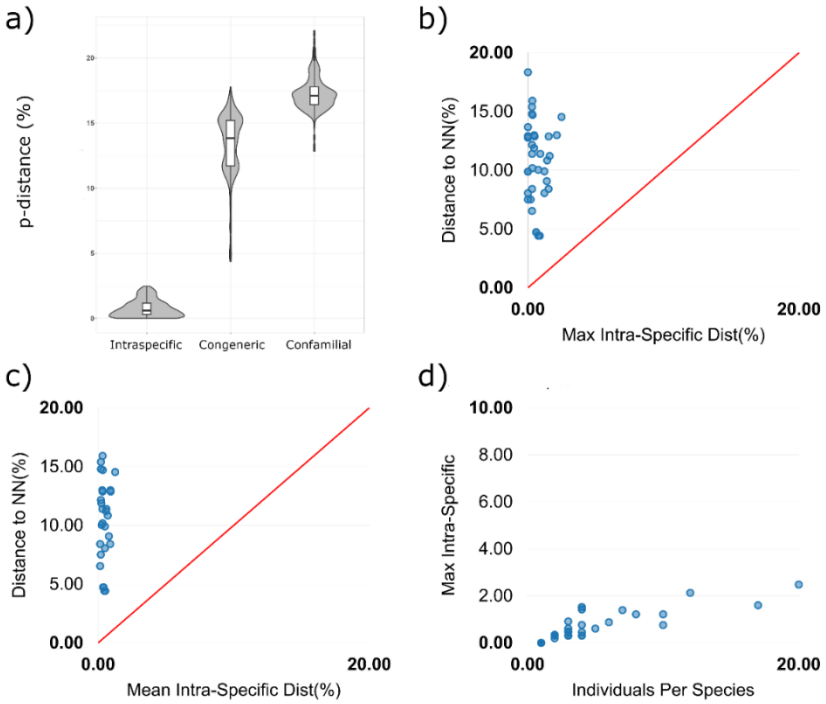

Figure 2. Distance boxplot and Barcode gap results. (a) Box plot of the p-distances at the species (intraspecific), genus (congeneric), and family level (confamilial); (b) The barcode gap shown by plotting maximum intraspecific distance against interspecific (nearest-neighbour) distance; (c) Scatterplot of the mean intraspecific distances against the minimum interspecific distances; (d) Scatterplot of the number of individuals in each species against their maximum intraspecific distances.

Discussion

The Plecoptera fauna of Romania was well explored by the thorough and detailed work of Kis Béla by the end of the twentieth century (Kis 1974). His detailed Fauna Book on the Stoneflies of Romania (Kis 1974) is a useful guide for the identification of the mature stonefly species of the country and is still a generally recognized and recommended reference work for the Carpathian stonefly fauna.

The Romanian stonefly checklist was later supplemented by additional data of Murányi Dávid, in his PhD thesis (Murányi 2008). Because of these studies, we have a good knowledge of the Plecoptera fauna of this country, mostly based on morphological data. With the development and availability of new genetic methods, molecular tools started to reform our general perception of taxonomy worldwide (Hebert & Gregory 2005). DNA-based new genetic tools proved to help species-level identifications (Ge et al. 2021), detect cryptic diversity (Zhou et al. 2010, Jackson et al. 2014, Cordero et al. 2017), and the identification of morphologically cryptic life stages (Fernanda et al. 2014, Gill et al. 2014, Molina et al. 2017), for many taxa. The situation is similar in Plecoptera, as well (Vitecek et al. 2017a, Ferreira et al. 2020, Hlebec et al. 2022, Laini et al. 2024, Vuataz et al. 2024). Despite the availability of these new methods, for Romanian Plecoptera species, there are just a few studies that apply molecular data, to reveal possible cryptic diversity (Bálint et al. 2011b) and describe new species (Murányi et al. 2014).

Despite the thorough morphological exploration of the Romanian Plecoptera, in addition to the biogeographic characteristic of the Carpathians as hotspot for aquatic fauna (Bálint et al. 2011a, Antal et al. 2016, Dénes et al. 2016, Copilaş-Ciocianu et al. 2018b) additional stonefly species are expected. The discovery of *Zwicknia kovacsi* Murányi & Gamboa, 2014 from Rodnei Mountains is a good example of this point of view (Murányi et al. 2014). Undetected new species are further expected to occur in less investigated habitats (high altitude headwaters), well-known biodiversity hotspots (ex. Bucegi Mts., *Zwicknia sp. n.* under description by the present authors), or under sampled periods (ex. late winter-early spring) (Dénes et al. 2021). Our study area, the Someşul Cald River headwaters from the Apuseni Mountains is one of the well-known biodiversity hotspots from Romania, with a considerable number of endemic species and highly

divergent genetic structures in a series of aquatic taxa (Bálint et al. 2011b, Dénes et al. 2016, Pârvulescu et al. 2020). The Plecoptera fauna of Apuseni Mts. is very rich, from the 132 species possibly present in Romania (the presence of 24 species is dubious), around half (71, 53.79%) were confirmed to this region, and more than the half of the endemic species of the country was found here (9 from the 14 endemic species, 64.29%) (Kis 1974, Murányi 2008), reinforcing the significance of this biodiversity hotspot in the case of stoneflies. In the present study we added new distribution data for the endemic *Leuctra carpathica* and the subendemic *Brachyptera starmachi* in the Apuseni Mountains. Up to now, these species have not been confirmed for this region. Among the endemic species, the region's stonefly biodiversity includes some rare species, with very restricted distribution area at a national level (ex. *Leuctra moselyi* Morton, 1929 and *Diura bicaudata* (Linnaeus, 1758) only in the Padiş region, Apuseni Mts., or *Leuctra transsylvanica* Kis, 1964 present in Romania only in the Apuseni Mts.) (Kis 1974).

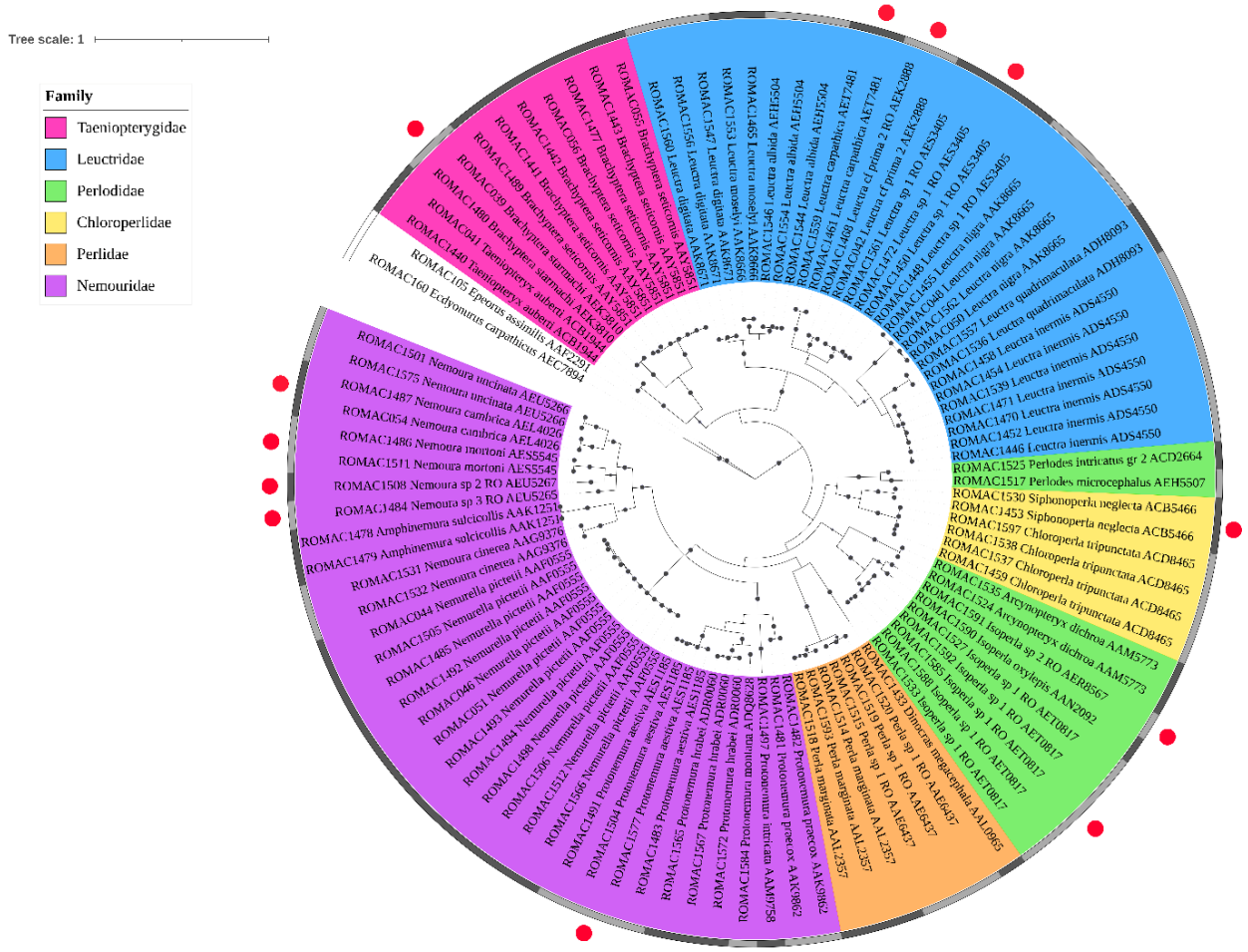

Figure 3. Bayesian inference tree based on the *mt*COI barcoding region. Sequences are color-coded by family. Dots on nodes represent Posterior probability values of 1. The grey outer circle shows the limit of each BIN. Red dots represent newly added BINs in the BOLD reference database.

We started building up the DNA barcode reference library of the Romanian stoneflies by creating barcode sequences for 36 morpho-species, that in 29 cases are known species, and in 7 cases represent the so called „dark taxa" (Page 2016), that up to now, based on morphological and molecular data, cannot be linked to any described species (Table 2.). Of the 36 BINs assigned to our dataset, 12 were new, belonging to known species or regional lineages, that did not have barcode sequences to date (to the best of our knowledge), or possible new species. These new molecular units, representing a third of the investigated taxa, emphasize the need of such integrative studies, especially in well-known biodiversity hotspots, like the Apuseni Mountains (Pârvulescu et al. 2013, Dénes et al. 2016, Szabó et al. 2023).

In seven cases, we could not match any known species to the specimens and molecular units. One is already known in the international genetic databases (BOLD). This taxon is designated *Perla sp. 1 RO* (BIN: AAE6437) and belongs to the *Perla pallida* species complex, which needs a comprehensive taxonomical review, suggested by egg morphology differences among populations from different geographical regions (Sivec & Stark 2002).

The additional 6 BINs are new records in the international databases (BIN: AEK2888, BIN: AES3405, BIN: AEU5267, BIN: AEU5265, BIN: AET0817 and BIN: AER8567). The first new BIN, designated *Leuctra cf. prima 2 RO* (BIN: AEK2888),

represents a cryptic lineage of *Leuctra prima*, based on the clear morphology of adult male specimens. Species similar to *L. prima*, such as the nearest neighbor of our new BIN, *Leuctra joani* Vinçon & Pardo, 1994 were described from the Atlantic Pyrenees. It shares similar morphology and ecology and was proven to be the result of recent speciation (Vinçon & Pardo 1994). The *Leuctra cf. prima 2 RO* collected in the Apuseni Mts. might also be a new, yet undescribed species of the same *Leuctra prima* group. According to Kis, 1974 *L. prima* is a frequent late winter-early spring species that can be found even on snow surface in the Apuseni Mts. and in the Eastern and Southern Carpathians. We managed to sequence one male specimen from the Eastern Carpathians that shares similar morphology with *L. prima*, but has significant genetic distance. This species was designated *Leuctra cf. prima 1 RO*, with a new BIN: AEK2886 (not published). These results suggest that the *L. prima* species complex should be investigated carefully among the biodiversity hotspots of the Carpathians.

The second new BIN, designated *Leuctra sp. 1 RO* (BIN: AES3405), is represented by 3 larvae and an adult female specimen, related to *Leuctra pseudosignifera* Aubert, 1954, a central European stonefly species, that also belongs to the *L. prima* species complex. It is frequently observed in Romania's mountain regions, according to Kis, 1974. The morphology of the adult female confirms the close relation to *L. pseudosignifera*, but the interspecific p-distance value (3.72%) suggests a considerable genetic distance between the species. Here we suggest a large-scale collection and detailed morphological and molecular analysis of *L. pseudosignifera*, to detect possible cryptic species and clarify the taxonomy of *L. prima* species complex.

The third and fourth new BINs, *Nemoura sp. 2 RO* (BIN: AEU5267) and *Nemoura sp. 3 RO* (BIN: AEU5265) are represented only by two larvae specimens, and both are related, as the nearest neighbor, to *Nemoura fusca* Kis, 1963, an endemic species of the Carpathians and Apuseni Mts. (Kis 1974). *Nemoura ovoidalis* Kis, 1965 is another species that has been found only in Romania (Eastern Carpathians and Apuseni Mts.), closely related to *N. fusca*. Our new molecular data may refer to this species or to unknown taxa that might represent other endemism of Apuseni Mts. and need further investigation by collecting additional adult specimens.

The fifth and the sixth new BINs, designated *Isoperla sp. 1 RO* (BIN: AET0817) and *Isoperla sp. 2 RO* (BIN: AER8567) are both genetically related to *Isoperla grammatica*. Even if we have adult male specimens in both cases, reliable morphological identification was not possible due to the damage of male genitalia. Even so, after examining male terminalia and head coloration of the specimens, we suspect that these taxa will be the BINs of two known species, that have not been sequenced yet, *Isoperla buresi* Raušer, 1962 and *Isoperla sudetica* (Kolenati, 1859). Additional specimens from these species will be sequenced in the near future as we aim to complete and further develop a more comprehensive reference library covering all species from Romania.

Overall, by sequencing adult specimens of one endemic (*Leuctra carpathica*) and two subendemic species (*Brachyptera starmachi, Protonemura aestiva*) from the Carpathians and Apuseni Mountains, we adjust the most general international databases (BOLD, NCBI) with valuable new data. We were also able to manage species-level identification of larvae of these range-restricted local species, making it possible for further studies to describe unknown larvae and adding new information about the ecology and habitat requirement of larvae of the species.

Studies from all across Europe support the efficacy of DNA barcoding for species determination; so, as a result, comprehensive barcode reference libraries of stoneflies were established for a few European countries (Morinière et al. 2017, Ferreira et al. 2020, Hlebec et al. 2022, Laini et al. 2024, Vuataz et al. 2024). These studies report a high success of species delimitation using well-established interspecific barcoding gaps (approx. 97%). However, in some particular cases, there is a need for additional molecular markers to support selected taxonomic hypotheses. Introgression, for example, was already documented in the case of *Leuctra* (Boumans & Tierno de Figueroa 2016) and *Zwicknia* (Boumans & Murányi 2014), that surely impacts the success of species delimitation. Thus, morphology and molecular data should be reexamined carefully based on a multiple-evidence methodology (Gattolliat et al. 2016).

The present study is the first integrative research on Romanian stoneflies, combining morphological and molecular data, through DNA barcoding. The total number of 29 stonefly species, included here, represents 22.66% of the total Plecoptera species from Romania (Fig.3), improving the bioassessment of freshwater habitats and our taxonomic knowledge, in the case of a reference headwater system, the Someşul Cald River. Within this study, we proved that barcoding methods could be applied to assessment and monitoring studies with a high sequencing success rate (90.86%). Our dataset shows that the distance to the NN is predominantly higher than the maximum intraspecific distance, resulting in a clear local barcoding gap (*p*-distance value of 2.49%, Fig. 2) (Pentinsaari et al. 2014), proving DNA barcoding a successful tool for the identification of stonefly species of the present study (Table 2.). All the 36 stonefly morpho-species form separated clades on our phylogenetic tree (Fig. 3.).

Although if we examine our data in relation with the nearest neighbor species, we find some cases of incongruence, that offer space for further studies: (1) when our species is identical to the NN species and (2) when the NN is not identical, but the interspecific p-distance is lower than the local barcoding gap. These cases of *mt*COI putative species lumping or splitting are explained by unrecognized species synonymy or species diversity, or by the lack of intermediate haplotypes of large unsampled geographic areas (Vuataz et al. 2024). In the case of 13 species (*Leuctra albida*, *Leuctra inermis* Kempny, 1899, *Amphinemura sulcicollis* (Stephens, 1836), *Nemoura uncinata* Despax, 1934, *Nemurella pictetii* (Klapálek, 1900), *Protonemura aestiva*, *Protonemura hrabei* Raušer, 1956, *Protonemura praecox* (Morton, 1894), *Perla marginata*, *Perla sp. 1 RO*, *Isoperla oxylepis* (Despax, 1936), *Isoperla sp. 1 RO*, *Siphonoperla neglecta*), where the observed interspecific p-distance was lower than the maximum of intraspecific distance (<2.49%) (Table 2.), we suspect two BINs representing the same morphospecies. In most of these cases, the NN species are similar to those identified by our work. When there are differences, like in the case of *Leuctra albida* – *L. meridionalis*, *Amphinemura sulcicollis* - *Amphinemura*

guadarramensis (Aubert, 1952), *Protonemura aestiva* – *Protonemura auberti* Illies, 1954, *Perla marginata* – *P. grandis*, *Isoperla oxylepis* – *I. grammatica*, we suppose possible misidentifications and suggest the need for further investigations, involving other molecular markers, more specimens and rigorous morphological analysis. In addition, in the case of five other species (*Leuctra nigra* (Olivier, 1811), *Nemoura cambrica*, *Nemoura cinerea* (Retzius, 1783), *N. mortoni*, *Protonemura intricata* (Ris, 1902)), where the nearest species seemed to be equal with the ones identified, but with the interspecific *p*-distance higher than the maximum of intraspecific distance (>2.49%) (Table 2.), we presume possible cryptic morphological diversity within species with large distribution areas.

An integrative approach of larvae identification, using COI barcodes in addition to classical morphology, can improve significantly the species-level identification of cryptic larvae forms (from 31.53% up to 88.65%), facilitating more detailed bioassessments of freshwater habitats through barcoding and metabarcoding (Table 1.). Of the 97 larvae specimens that we managed to sequence, more than half (67.01%) corresponded to 15 species through association with adult specimens sequenced by the present study, and only a small portion (18.55%) was identified by using barcode sequences generated by other works. This result confirms the need for a Romanian barcode reference library of stoneflies containing sequences from local populations.

Although adult stoneflies from Romania can be distinguished quite accurately by morphology (Kis 1974), to detect cryptic biodiversity and even new species to science we need to combine morphology with molecular data (Cordero et al. 2017, Vitecek et al. 2017a), to get a more detailed dimension of the diversity of stoneflies in Romania. Because of our work, molecular data for endemic and subendemic species are available, and regional lineages, whose larvae can now be detected more accurately, by applying barcoding methods. Thus, ecological research and conservation actions can be made to protect their habitats, an important aspect in the age of constant biodiversity loss among freshwater habitats (Sayer et al. 2025). The present work, is the first milestone towards a DNA barcode reference library for Plecoptera of Romania, which raises the need for further research on "dark" taxa that based on our current data, we could not link to any existing species, and supports the Apuseni Mountains as a biodiversity hotspot of unknown biodiversity also for Plecoptera.

From a more practical view, comprehensive and well-populated DNA reference libraries make species-level identification a routine task for non-expert practitioners in water conservation and management, helping sustainable preservation of the unique aquatic biodiversity from here.

DNA barcode reference libraries are very important nowadays, especially given the significant lack of taxonomic experts who can provide high-quality species-level identifications. At the same time, detailed taxonomic data is essential for comprehensive bioassessments and effective conservation actions. Our study is the first initiative to establish a validated DNA barcode reference library for stoneflies in Romania, supporting cost-efficient identification and bioassessments while providing molecular characterization of endemic and subendemic species and regional lineages. Regarding the unknown taxa detected during the present study, we focused attention on the need for further collection and analyses covering the complete biogeographic area of the Carpathians and more. It proved clear that, due to cryptic morphological diversity, endemic, and regional species, we need local datasets to support regional aspects of the taxonomy of Plecoptera from here. Biodiversity hotspots, such as the Apuseni Mountains, should be in the focus while establishing this database, to reveal a more realistic dimension of stonefly diversity in Romania.

The usefulness of our molecular data is particularly outstanding in the species-level identification of different morphologically cryptic stonefly larvae by simple genetic association with the already-known corresponding adult forms. This genetic tool is essential in improving biological assessments of freshwaters, where only larvae are present in samples, and the taxonomic accuracy of identification based on morphology is very low (see our comparative data).

We aim to continue this project until we can complement our database with DNA barcode sequences for all the known stonefly species in Romania in the near future.

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