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DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

The Carpathians as cumulative refugia: case studies of relict-endemic crane fly (Diptera: Pediciidae) groups

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Abbreviations

AMOVA	Analysis of the Molecular Variance
BI	Bayesian Inference
BIN	Barcode Index Number
BOLD	Barcode of Life Data System
bp	base pair
BP	Bootstrap
CCW	Catalogue of the Cranfly of the World
COI	Cytochrome *c* oxidase I
GTR	General Time Reversible model
KOH	Potassium Hydroxide
LGM	Last Glacial Maximum
MJN	Median Joining Network
ML	Maximum Likelihood
MOTU	Molecular Operational Taxonomic Units
*mt*COI	mitochondrial Cytochrome *c* oxidase I
*mt*DNA	mitochondrial DNA
Mts	Mountains
Mya	Million years ago
OTU	Operational Taxonomic Units
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PP	Posterior Probability
SD	Standard Deviation

Keywords:

BOLD, Cryptic diversity, integrative taxonomy, mtCOI, speciation.

I. Introduction

1. The Carpathians as an important refugia and speciation center

1.1. Mechanisms that shaped Europe's biodiversity

For several decades the Quaternary glaciations were considered as the most important mechanism that influenced the European divergence and speciation processes. However, many of the European endemic species have a relict-like character and can be dated back to the Miocene or Pliocene periods. The origin and evolution of such species is related with the Alpine orogenic events in central Europe, or the repeated transgression and regression phases of the Paratethys Sea in the Miocene, with the aridization that led to forest fragmentation (Habel & Assmann, 2010), or the volcanism that caused isolated enclaves resulting in accelerated insular-like speciation in the Pliocene (Pop *et al.*, 2010).

The existence of several cryptic refugia to the north, outside of the traditional Mediterranean region, was confirmed by several molecular and morphological results (Bálint *et al.*, 2011), showing that the cold stenotherm aquatic environments of the deep headwater and river valleys provided stable, buffering climatic conditions making the survival possible even in the harsh conditions of the glaciation maximum periods (Schmitt & Varga, 2012).

1.2. The importance of the Carpathians in a biogeographic context

The importance of the Carpathians Mountains mountain range in the biogeographical context of the European diversity is due to its geographical position, size, lower altitude, fragmentation and geomorphological complexity. Because of the central European position, the biota of this mountain range was influenced by the alpine, arctic, Mediterranean and even Asian regions. The high number of relict-like autochthonous organisms and old lineages can be correlated with the island-like isolation of the different mountain ranges (Dénes *et al.*, 2016a).

The species belonging to the Pediciidae family inhabit semi-aquatic habitats along the mountain streams (Keresztes *et al.*, 2011). Because they are mostly limited to higher altitudes, they also show insular-like distributions and can be interesting model organisms for studying the evolutionary history of the Carpathian semi-aquatic species.

2. General characterization of the crane flies (Diptera, Tipuloidea) with emphasis on the Pediciidae family

2.1. Tipuloidea – general remarks

The crane flies (Tipuloidea) are one of the most diverse Dipteran groups with 15527 species belonging to 709 genera and subgenera of four families (Oosterbroek, 2019). They are a cosmopolitan, widespread group, with a distribution ranging from the arctic region to the equator,

and from the marine tidal zones to the high mountains, up to 5600 m in certain regions (Alexander & Bayers, 1981).

2.2.Classification of Tipuloidea

The first integrative approach to crane fly phylogeny was done by Petersen *et al.* (2010), who combined the analyses of one hundred morphological characters of the larvae, pupae and adult, with the sequence analysis of the 28S rRNA gene and the CPS region of CAD. The results divided the Tipuloidea into two families: Pediciidae and Tipulidae.

2.3.The Pediciidae family

Oosterbroek (2006) describes a number of specific morphological features that distinguish Pediciidae family members from other species. Their size is very variable, from very small 5 mm of some *Dicranota* species, to a much larger body of 35 mm of species belonging to the *Pedicia* genus. Their body, antennae, legs and wings are elongated and long, as is characteristic to almost all members of Tipuloidea. Short hair, ommatidia is present between the eye units in all members of the family, that is why they are also called "hairy-eyed" crane flies. Their wing venation also has several distinctive characteristics (Dienske, 1987).

2.4.Pediciidae in Romania and the Carpathians

In 1998, Ujvárosi started a comprehensive research to assess the fauna of the hairy-eyed crane flies of the Carpathians in Romania and found that the most suitable habitats for the Pediciidae in the Carpathian Mountain range are the wetlands and swamp areas covered by forests where there is a high diversity with abundant communities, and the headwater regions where several rare endemic species can be found (Ujvárosi, 2005). This is confirmed by the description of *Pedicia apusenica* from the Apuseni Mountains (Ujvárosi & Starý, 2003). In 2010 Ujvárosi *et al.* found two divergent lineages of *Pedicia occulta* (Meigen, 1830) in the Carpathians, which led to the description of a new species, the *Pedicia fusca* based on morphological and molecular methods (Ujvárosi & Bálint, 2012). Combining the morphological data and the analysis of the COI sequences, cryptic diversity was found in the case of the *P. staryi* species group (Dénes *et al.*, 2016a,b).

3. The mitochondrial cytochrome oxidase I gene as a tool of molecular taxonomy and phylogeography.

3.1.Mitochondrial markers

Mitochondrial DNA (mtDNA) markers are one of the most widely used sequence-based tools in molecular taxonomy and phylogenetic studies. These markers have several qualities that can explain their popularity, from their large number of copies present in one cell, which makes it

relatively easy to isolate and analyze, to the maternal inheritance (Sato & Sato, 2013), and the relative rapid evolution rate (Avise, 2009).

3.2.The Barcoding of Life System

In 2003, Hebert *et al.* proposed 648 base pairs (bp) sequence of the cytochrome *c* oxidase I (COI) mitochondrial gene as the suitable universal marker for metazoans, because it is one of the most conservative mitochondrial protein coding gene therefore there are several robust universal primers that are used in taxonomic and phylogenetic studies in a large spectrum of animal phyla (Folmer *et al.*, 1994), making it a suitable choice for universal use, leading to the successful Barcode of Life Data System (BOLD, http://www.boldsystems.org; Sujeevan & Hebert, 2007).

A multi-step algorithm called "Refined Single Linkage (RESL) Analysis" (Ratnasingham & Hebert, 2013) is used to assign every sequence that was grouped together after these steps to a Barcode Index Number (BIN). The system also takes the morphology based taxonomic units into consideration and recalculates the thresholds with every newly added sequence (Ratnasingham & Hebert, 2013).

II. Aims of the study

The most important aim of this thesis is to confirm the importance of the Carpathian mountain system as cumulative refugia and biodiversity hotspot for the crane flies belonging to the Pediciidae family (Diptera). Studies focusing on this question in other insect groups from the region are still scarce, although the existing biogeographic works suggest the importance of the Carpathians as a biodiversity center for the European biota. The morphology-based studies and the number of endemic aquatic and semi-aquatic species suggested the need of a more integrative approach that also include the study of molecular genetic diversity. The molecular genetic analyses in the thesis are based on the *mt*COI barcode sequences and are discussed in two case studies focusing on three Pediciid groups. In the first study we analyze the molecular and morphological differentiations and the cryptic diversity of the *Pedicia* (*Crunobia*) *staryi* Savchenko, 1978 species complex. In the other case we focus on the *Dicranota* genus, specifically on the diversity observed within the *Paradicranota* Alexander, 1934 subgenus and a species of the *Ludicia* Hutson and Vane-Wright, 1969 subgenus, the *Dicranota* (*Ludicia*) *lucidipennis* (Edwards, 1921).

As the marker used in our molecular genetic studies was the *mt*COI barcode sequence, another aim of this thesis was to test the utility of this marker in testing taxonomic hypothesis, checking the position of the *Ludicia* (*Dicranota*) subgenus, a group with debated taxonomic position, within the Pediciidae family.

III. Materials and methods

1. Specimen collection

Specimens used in this study were collected using sweep nets or by hand along springs and headwaters and were stored in 96% ethanol in the Diptera Collection of the Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania. A total of 360 individuals were collected from running water localities all over Europe, from May to August between 2007-2018 and were analyzed in two separate case studies.

2. Molecular methods

The sequences analyzed in this thesis were acquired, in two ways. The laboratory work and the sequencing for a large number of individuals were done as part of the BOLD system in the Canadian Centre for DNA Barcoding, University of Guelph, Guelph, Canada. Additional sequences were produced as a result of the work performed in the Interdisciplinary Research Institute on Bio–Nano–Sciences of Babeș–Bolyai University, Cluj-Napoca, Romania.

2.1.BOLD System sequencing

All specimens were photographed and were uploaded to the BOLD system, together with additional information regarding their taxonomic classification and collection data. Two or three legs were collected from each individual and were loaded in 96-well plates containing 30 µl of 96% ethanol, which were sent for sequencing to the Canadian Centre for DNA Barcoding (Wilson, 2012). Sequences and trace files became available through the BOLD Systems (http://www.boldsystems.org) under the Tipuloidea of Europe [EUTIP] project name, after successful sequencing.

2.2.Genomic DNA extraction

DNA was extracted from thorax-tissue samples using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the provided standard spin-column based extraction protocol for animal tissue. Genomic DNA purity and concentration was checked with a NanoDrop 1000 fiber-optic spectrophotometer.

2.3.Amplification of the *mt*COI sequence

The *mt*COI sequences were amplified in a 50 µl reaction volume using the LCO1490/HCO2198primer pair.

2.4.PCR product purification

The PCR products were loaded onto a 1% agarose gel, the target fragment was cut off and purified using a Wizard SV Gel and PCR Clean–Up System (Promega, USA).

2.5.Sequencing

The PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands) for sequencing using the LCO1490 standard primer.

2.6.Sequence analysis

Sequence chromatograms were visualized with Trev (Staden Package Program; Bonfield *et al.*, 2002) for quality check and were corrected by hand. All fragments were aligned in BioEdit version 7 (Hall, 1999) with the Clustal W multiple alignment algorithm,

2.7.Molecular data analysis

The basic sequence analysis for each dataset, including number of polymorphic sites, the number of haplotypes, haplotype (h) and nucleotide (p) diversities were obtained with DnaSP, version 5 (Librado & Rozas, 2009).

3. Morphological methods

3.1.Morphological variability analysis

The male genitalia were macerated in 10% (KOH) for 10-12 minutes to relax the sclerotized parts and open the genital structures. They were placed on a bed of fine glass under glycerol and were analyzed using an Olympus SZ61 stereomicroscope equipped with a Canon 650D camera and an LM Digital SLR Adapter (Micro Tech Lab, Austria).

3.2.Morphometrical measurements and data analysis

Micromorphological measurements were carried out in Gimp 2 software (www.gimp.org) based on the photos of male genitalia or wings. The terminology of the characters is based on Dienske (1987).

IV. Complex evolutionary history in the Carpathian Area based on the diversity and distribution of the micro endemic *Pedicia* (*Crunobia*) *staryi* species complex

1. General remarks

The biodiversity of a region and the number of endemic taxa is influenced by the geological age of that region and reflect the past and present local conditions characterizing it (Varga, 2010). Therefore, cold adapted semi-aquatic species as the members of the *Pedicia* (*Crunobia*) *staryi* species complex are good model organisms for studying and understanding the historical processes that influenced the diversity of mountain systems in relatively stable environment (wet and humid environment of headwater springs) over larger periods than the Pleistocene glaciations.

1.1.The studied *P. staryi* species group

The *Pedicia* (*Crunobia*) *staryi* species complex was established by Savchenko in 1986, who differentiated the '*littoralis*' species group as having only two thorns on the gonostylus (only *P. nielseni* has three) and grouped the other species, with more than two such spines to the '*staryi*' group. Previous to the work presented in this thesis, the classification schemes of this species complex was represented by five taxonomic units: *Pedicia* (C.) *apusenica* Ujvárosi & Starý, 2003, *P.* (*C.*) *lobifera* Savchenko, 1986, *P.* (*C.*) *staryi* Savchenko, 1978, *Pedicia* (*C.*) *spinifera* Starý, 1974 and *P.* (*C.*) *straminea* Meigen, 1838. *Pedicia lobifera, P. staryi* and *P. apusenica* are narrow endemics in the Carpathians, and the Apuseni Mountains. In the Bulgarian Mountains they are replaced by *P. spinifera*. The last member of this group, *P. straminea* is widely distributed in various headwater habitats at different altitudes in Europe (Oosterbroek, 2019).

1.2.Aims of the study

The aim of this case study is to analyze the molecular and morphological variation within and between species of the *Pedicia stary* complex, to identify potential cryptic diversity, focusing mainly on the Carpathian endemic species. It is also to study the importance of the Carpathian Mountains as refugia and speciation centers by analyzing the evolution history of these species.

2. Materials and methods

152 individuals of the *P. staryi* species group were used in this study:83 individuals of *P. staryi*, 17 of *P. apusenica*, 9 of *P. lobifera*, 6 of *P. spinifera* and 37 of *P. straminea*.

2.1.Molecular data analysis

Sequences were obtained from the BOLD system or generated through the laboratory work in the Interdisciplinary Research Institute on Bio–Nano–Sciences of Babeș–Bolyai University as presented in the general description of the methods.

2.1.1. *Spatial genetic structuring*

The spatial clustering of individuals was implemented in the case of the endemic autochthonous Carpathian species of the *P. staryi* group. BAPS, version 6 (Corander, Sirén, & Arjas, 2007) was used to detect population sub structuring and to identify the main haplogroups of the three Carpathian endemic species of the species complex.

2.1.2. *Molecular genetic diversity*

A hierarchical analysis of the molecular variance (AMOVA; Excoffier, Smouse, & Quattro, 1992) was implemented in Arlequin, version 3.5 (Excoffier & Lischer, 2010). Populations were grouped into two groups corresponding to the current taxonomic status, and into five groups suggested by the population structure analysis. The proportion of nucleotide differences, the *p*-distance was also calculated between the a priori groups, using Mega X (Kumar *et al.*, 2018).

2.1.3. *Phylogenetic analyses*

The phylogenetic relationships were inferred with a Maximum Likelihood (ML), a Bayesian inference (BI) algorithm and a Median Joining Network (MJN).

2.1.4. *Divergence time estimation*

BEAST, version 1.7.4 (Drummond & Rambaut, 2007) software using a Bayesian Markov chain Monte Carlo (MCMC) method, with the Yule-type speciation process (Steel & McKenzie, 2001), was implemented to infer the phylogeny and the divergence time of the nodes. Due to the absence of closely related fossil records, a value of 0.0177±0.00119 was employed as lineage substitution rate (the Mid-Aegean trench calibration Papadopoulou *et al.*, 2010).

2.2.Morphological methods

2.2.1. *Morphological variability analysis*

The morphological appearance of the individuals and morphological characteristics of the male terminalia were examined as described previously in the general “Methods” chapter.

2.2.2. *Morphometrical measurements and data analysis*

In the case of the *P. staryi* species complex, besides the morphological analysis, micromorphological 11 morphological characters were measured on the male genitalia. Pairwise comparison of the measured morphological variables was made with Mann-Whitney U test in SPSS Version 17.0. (Chicago: SPSS Inc). A principal component analysis (PCA) with the prcomp function from the built-in R stats package, was calculated and plotted in R (R Core Development Team, 2016).

3. Results

3.1. *mt*COI sequencing results

The COI alignment was 658 base pair (bp) long with a total number of 471 sites after excluding the gaps and missing data. Of the 130 polymorphic positions 129 were parsimony informative resulting in 44 haplotypes with 0.938 haplotype diversity and 0.08633 nucleotide diversity.

3.2. Spatial clustering and genetic diversity within and between the endemic Carpathian species of the group

BAPS defined six groups (optimal partition, log(likelihood) = -3578.72). *P. lobifera* and *P. apusenica* form two well separated groups, with *P. lobifera* distributed in the Eastern Carpathians and *P. apusenica* present only in the Apuseni Mts. The *P. staryi* individuals form four separate genetic groups with two haplogroups from the Rodnei Mts (*staryiR1*; *staryiR2*), a third present in the Gutâi Mts (*staryiG*), and a last one from Bucegi Mts (*staryiB*).

AMOVA showed the highest amount of variation when the *a priori* grouping was based on the five structures suggested by the BAPS analysis with the highest diversity explained by the variation among the five groups (79.55%), followed by the variation at the population level (15.05%). The highest *p*-distance is 9.10% between *staryiR1* and *staryiB*, followed by the distance between *staryiR1* and staryiR (8.98%). All distances are higher than the 2% considered as universal species delimitation boundary, with the lowest *p*-distance value of 4.86% between *staryiR1* and *staryiG.*

3.3. Phylogenetic analysis

Both ML and BI tree constructions resulted in congruent tree topologies showing a monophyletic *P. staryi* species group but without a strong support. All lineages are well separated and show strong support.The shortest MJN had 153 estimated numbers of mutations and confirm the presence of the four well separated lineages of *P. staryi* (Fig. 1).

3.4.Micromorphological differentiation

The PCA show three well separated groups (*staryiR1*, *staryiG* and *staryiR2*) based on the first and second component. The individuals corresponding to the *staryiB* lineages overlap with the other three groups, but are well separated based on other characters, as every measured character of the male terminalia showed significant differences between one or more groups of the *P. staryi* species.

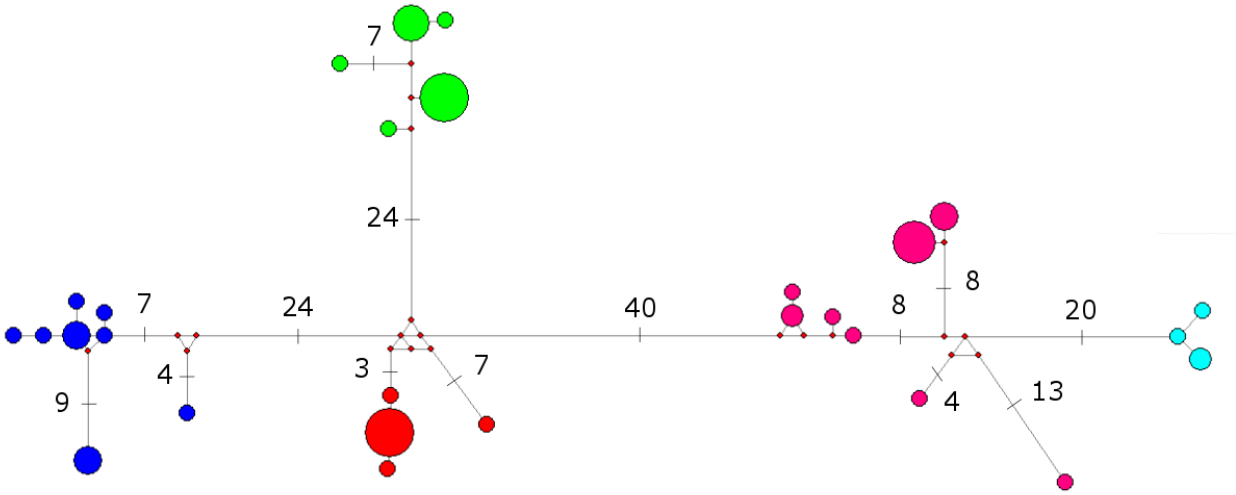

Fig. 1. MJN; circles represent the different haplotypes; numbers on the branches show the mutational steps between the haplotypes; colors represent the five groups: *P. apusenica* (red), *staryiG* (blue), *staryiR1* (purple), *staryiR2* (green), *staryiB* (dark blue).

3.5.Description of the differentiated taxonomic units

All these results suggested the need of a taxonomic revision, therefore *P. staryi* was redefined as a species corresponding to the *staryiR2* lineage an tdhe other three lineages were described as new species in Dénes *et al.* (2016b).

3.5.1. *Pedicia* (*Crunobia) apusenica* Ujvárosi and Starý, 2003

BOLD accession number: EUTIP718 to 720 and EUTIP725. BIN: AAF8237

Large species of a yellowish orange color (Fig. 2-A). **Male** body length is 14–15 mm, (mean 14 mm, n = 20), wing length 13–15 mm (mean = 14.1 mm, n = 20), antenna 1.9–2.1 mm (mean 1.95, n = 9). **Female** has a body length of 15 mm; wing length 9 mm.

3.5.2. *staryiR2* – redescribed as *Pedicia* (*Crunobia*) *staryi* Savchenko, 1978

Gen Bank accession number: KT983907 to KT983910; BOLD accession number: EUTIP709. BIN: ACL4087.

Large species having general color yellowish orange (Fig. 2-B). **Male** body length is 13–16 mm, (mean 14.2 mm, n = 20), wing length 13–15 mm (mean = 14.1 mm, n = 20), antenna 1.9–2.1 mm (mean 1.95, n = 9). **Female** body length is 16.5–17 mm, wing length 12–13 mm, antenna 1.7 mm.

3.5.3. *staryiR1* – described as *Pedicia* (*Crunobia*) *carpianica* Kolcsár, Keresztes & Dénes 2016

GenBank accession number: KT983904 to KT983906; BOLD accession number: EUTIP095, EUTIP096, EUTIP475, EUTIP478 and EUTIP480. BIN: AAD6568, AAD6569 ABA7405, ABA7406.

Large species with yellowish orange color (Fig. 2-C). **Male** body length is 13–17 mm, (mean 15.4 mm, n = 13), wing length 13.5–17 mm (mean = 15.4 mm, n = 13), antenna 1.9–2.1 mm (mean 1.98, n = 7). **Female** is unknown.

3.5.4. *staryiG* – described as *Pedicia* (*Crunobia*) *costobocica* Kolcsár, Keresztes & Dénes 2016

BOLD accession number: EUTIP695, EUTIP698 and EUTIP708. BIN: ACL4088.

Medium sized species of a yellowish orange color (Fig. 2-D). **Male** body length is 10–14 mm, (mean 12.9 mm, n = 8), wing length 11–14.5 mm (mean = 13 mm, n = 8), antenna 1.7 mm (mean 1.7, n = 5). **Female** has a body length of 12 mm, wing length 11 mm, antenna 1.6 mm. General color is yellowish.

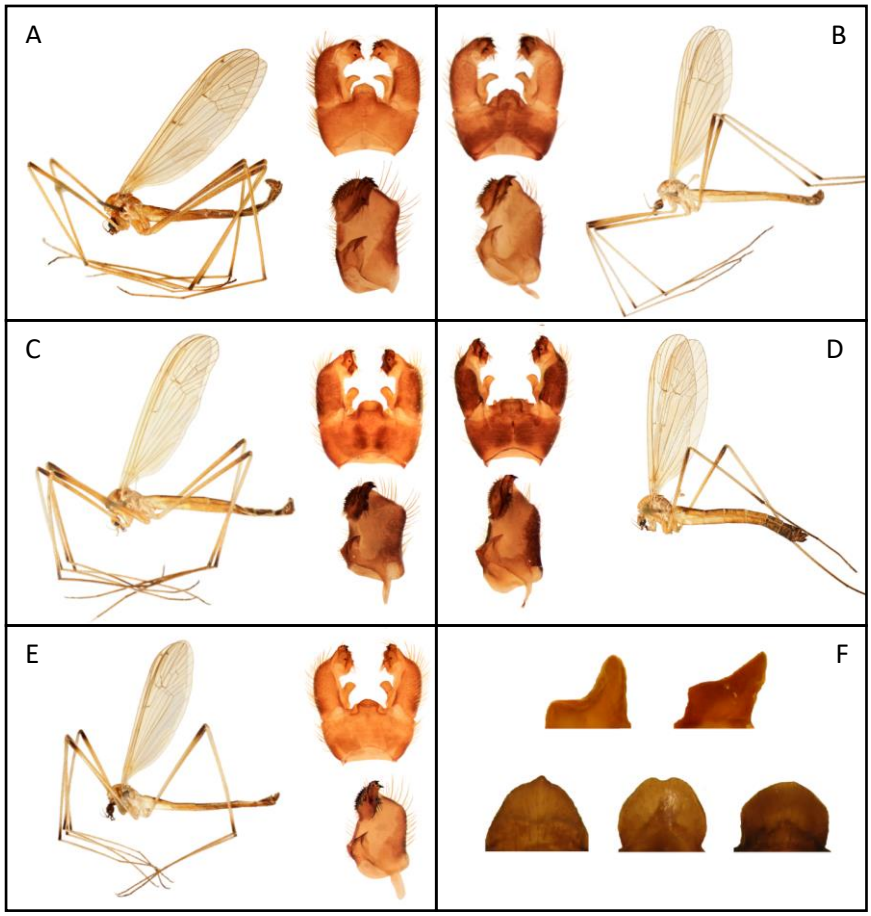

Fig. 2. Male adult lateral habitus, inner lateral gonocoxite view and hypopygium dorsal view: *P apusenica* (A); *P. staryi* (B); *P. carpianica* (C); *P. costobocica* (D); *P. roxolanica* (E). The F panel: gonocoxite lobe from lateral view (upper row) of *P. roxolanica* (left) and the rest of the species (right) and the lobe from dorsal view of *P. apusenica* (left) and the other species of the group (middle and right). Photos A to E by Levente Péter Kolcsár.

3.5.5. *StaryiB* – described as *Pedicia* (*Crunobia*) *roxolanica* Kolcsár, Keresztes & Dénes 2016

Gen Bank accession number: KT983903; BOLD accession number: EUTIP669, EUTIP670, EUTIP691 to 694 and EUTIP710. BIN: AAD6568.

Large species of a yellowish orange color (Fig. 2-E). **Males** body length is 13–15 mm, (mean 14.2 mm, n = 7), wing length 13–14.5 mm (mean = 13.9 mm, n = 7), antenna 1.6–1.9 mm (mean 1.7, n = 5). The head has vertex yellowish orange to dark brown. **Female** is unknown.

3.6.Divergence time estimation

Molecular dating estimates that *P. spinifera* appeared approximately 10.16 Million years ago and the common ancestor of the autochthonous Carpathian species diverged from *P. straminea* 8.65 Million years ago. It further shows that *P lobifera* split 7.10 Mya from the other five endemic species. The clade formed by *P. carpianica* and *P. costobocica* diverged from that of the other three species around 5.81 Mya, with *P. carpianica* and *P. costobocica* separating approximately 2.65 Mya. *Pedicia apusenica* and *P. roxolanica* diverged from *P. staryi* 4.80 Mya, and they split 4.07 Mya.

4. Discussions

4.1.Phylogenetic relationships within the *P. staryi* species group

The three newly described species, *P. costobocica*, *P. carpianica* and *P. roxolanica* can clearly be attributed to the '*staryi*' species group *sensu* Savchenko (1986) because they have more than two black spines on the top of the gonostylus. In concordance with Savchenko's (1986) taxonomic hypotheses, the Maximum Likelihood and Bayesian Inference phylogenetic analyses based on mitochondrial DNA sequence data also support the *P. staryi* group as a monophyletic unit.

4.2.Molecular genetic divergence in the Carpathians

The ancestor of the Carpathian endemic species diverged about 8.65 Mya from *P. straminea*, when due to the subtropical conditions, cold adapted species were restricted to forest patches in the mountain regions (Kvaček *et al.*, 2006). *Pedicia lobifera* diverging about 7.1 Mya. This event can be explained with another aridification (van Dam, 2006). It can also be the result of the isolation in an insular enclave during the transgression of the Paratethys (Pop *et al.*, 2010). The diversification of the other five lineages started at the beginning of the Messian salinity crisis, about 5.81 Mya, when the lineage of *P. carpianica* and *P. costobocica* split from the lineage of *P. apusenica*, *P. staryi* and *P. roxolanica*. The split between *P. staryi* and the clade formed by *P. roxolanica* and *P. apusenica* happened about 4.80 Mya. *Pedicia roxolanica* and *P. apusenica* diverged approximately 4.07 Mya. Climatic conditions of the Carpathian region where dry in this

period, but seasonally homogenous (van Dam, 2006). At the end of the Pliocene the global climate started cooling, resulting in the beginning of the glaciations, thus the split between *P. carpianica* and *P. costobocica* lineages (2.88 Mya) is probably the result of the Late Pliocene glaciations.

4.3.The cumulative nature of refugia in the Carpathian

Most of the newly discovered microendemic species of the Carpathians are highly specialized rithral elements concentrated near the sources of cold stenotherm springs, showing an important degree of still undiscovered diversity. The cumulative pattern and the distribution of such range restricted endemics underline the importance of some mountains ranges in preserving the present autochthonous aquatic diversity. Particular centers for diversification hosting several endemic species in the Carpathians are the northern part of the Eastern Carpathians (Czarnahora-Maramures-Rodnei), the Southern Carpathians (Bucegi Mountains) and the Apuseni Mountains.

V. Revision of the *Dicranota* Zetterstedt, 1838 (Diptera, Pediciidae) genus in the Carpathian area

1. General remarks

Aquatic insects are known to present high degrees of "insular-like" endemism in the European Alpine system, with a considerable number of cryptic species, due to the selective pressure of the aquatic environment and insular-like distribution of the available habitats (Bálint *et al.*, 2011). Thus, consequent revisions of aquatic taxa are required based on important molecular divergencies at a range-wide context (Dénes *et al.*, 2016a).

1.1.The studied *Dicranota* Zetterstedt, 1838 genus

Due to the lack of molecular based revisions, a number of conflicting morphology based classification schemes were proposed for *Dicanota*. In this study the classification proposed by Oosterbroek's Catalogue of the Craneflies of the World (CCW; 2019) is used, where a number of 11 subgenera are recognized so far, from which only 5 are present in the Western-Palearctc area: *Dicranota* Zetterstedt, 1838 with 4 species; *Ludicia* Hutson and Vane-Wright, 1969 with 4 species; *Paradicranota* Alexander, 1934 with 29 species; *Plectromyia* Osten Sacken, 1869 with 1 species and *Rhaphidolabis* Osten Sacken, 1869 with 1 species, respectively.

1.1.1. *The subgenus Ludicia Hutson and Vane-Wright, 1969*

The subgenus *Ludicia* was delimited based on the characters of *Tricyphona* (*Amalopis*) *lucidipennis* known today as *Dicranota* (*Ludicia*) *lucidipennis* (Edwards, 1921). Brindle (1963) transferred *D.* (*L.*) *claripennis* (Verrall, 1888) and *D.* (*L.*) *lucidipennis* (Edwards, 1921) to the *Dicranota* genus based on wing venation, characters of the hypopigium and larval characteristics. Vane-Wright (1969) erected this group to a subgenus level, pointing out that based on the characteristics of the adult specimens, they differ both from *Pedicia* (*Tricyphona*) and Dicranota *sensu lato*.

Dicranota (*Ludicia*) *lucidipennis* was described by Edwards in 1921 as *Tricyphona lucidipennis* based on generally black colour and external morphology of the adult specimens. Later the close related *Pedicia (Tricyphona) luteicolor* was described from the Balkans by Alexander in 1975, having a lighter, brownish colour, and also some differences on the male genital structures. Based on the re-examination of the male holotype of *P. (T.) luteicolor*, Starý (2007) considered that the species is identical with *D. (L.) lucidipennis*, Quite recently an even lighter, yellowish form was collected for the first time in the Carpathians in sympathry with the dark colored form (Kolcsár *et al.*, 2014) showing the necessity of testing taxonomic hypotheses of *D. lucidipennis* using a molecular DNA approach.

1.1.2. *The subgenus Paradicranota Alexander, 1934*

Species belonging to *Paradicranota* are differentiated from all other Pediciidae based on the small size, frequently between 5-8 mm and antennae with only 10-11 short flagellomeres in both sexes. Wing clear, without macrothrichia, but supernumerary crossveins present in cell r1 and pterostigmal spot weakly developed and hypopygium with more or less developed lateral process on the 9th tergite and a well-developed apical lobe of the gonocoxite. The outer gonostylus near strong setae, interbasis of diferent shapes.

1.2. *Aims of the study*

The aims of this case study were to asses the utility of the barcode sequences to test taxonomic hypotheses whitin the *Dicranota* genus, mainly focusing on the position of the *Ludicia* subgenus within the Pediciidae family. The identification of cyiptic diversity based on the *mt*COI sequences was also an important aim of this analysis, mainly to test the "refugia within refugia" paradigm in case of "insular-like" range restricted populations of *Dicranota* in the Carpathian area.

2. Materials and methods

A total of 221 individuals were used in this study.

2.1.Molecular data analysis

Sequences were obtained from the BOLD system or generated through the laboratory work in the Interdisciplinary Research Institute on Bio–Nano–Sciences of Babeș–Bolyai University as presented in the general description of the methods.

2.1.1. *Phylogenetic analyses*

The phylogenetic relationships between the subgenera and species of the genus *Dicranota* were inferred with the Bayesian inference (BI) and the Maximum likelihood (ML) algorithms.

For *D. (L.) lucidipennis* besides the two tree inferring methods, a MJN was also calculated using NETWORK, version 4.6.1.0 (Bandelt, Forster, & Röhl, 1999) to analyze the relationship of the different BINs.

2.1.2. *Molecular genetic diversity*

The proportion of nucleotide differences, the *p*-distance was calculated between the *Paradicranota* species, to verify the divergence of the ABA7291 BIN. This distance was also calculated for the major clades shown by the phylogenetic trees in the case of *D. (L.) lucidipennis* using Mega X (Kumar *et al.*, 2018).

2.2.Morphological methods

2.2.1. *Morphological variability analysis*

The morphological appearance of the individuals and morphological characteristics of the male terminalia were examined as described previously in the general Methods chapter.

In the case of *D. (L.) lucidipennis* the hypopigium of 260 specimens were thoroughly analyzed to identify potential unrecognized variations.

3. Results

3.1.Phylogeny of the genus *Dicranota* Zetterstedt, 1838

The BI tree (Fig 3) shows *Ula* to be the oldest genus of the Pediciid clades. The *Tricyphona* species group together in a well-supported (PP = 1) clade, that cluster together with the only *Pentacyphona* species (although with low support: PP = 0.55), providing the root for the polytomous group of *Pedicia* + *Dicranota*. The *Dicranota* genus is the third well differentiated, monophyletic clade of the polytomy (PP = 1). The three genera represented in this study form well defined taxonomic groups, with strong support. *Dicranota claripennis* and *D lucidipennis*, the representatives of the *Ludicia* subgenus, are grouped in the oldest monophyletic clade of the genus (PP = 0.99). *Dicranota lucidipennis* is represented by nine BINs, showing several well differentiated lineages that will be further analyzed in a later section.

The subgenus *Dicranota* is represented in this study only by *D. bimaculata*, which is the sister clade of the *Paradicranota* species (PP = 0.99). Due to the presence of several BINs for one species (see for example *Dicranota* (*Paradicranota*) *flammatra* Starý, 1981) or the BIN (ABA7291) that does not belong to any described species, another tree was generated for this subgenus and will be discussed in the next section.

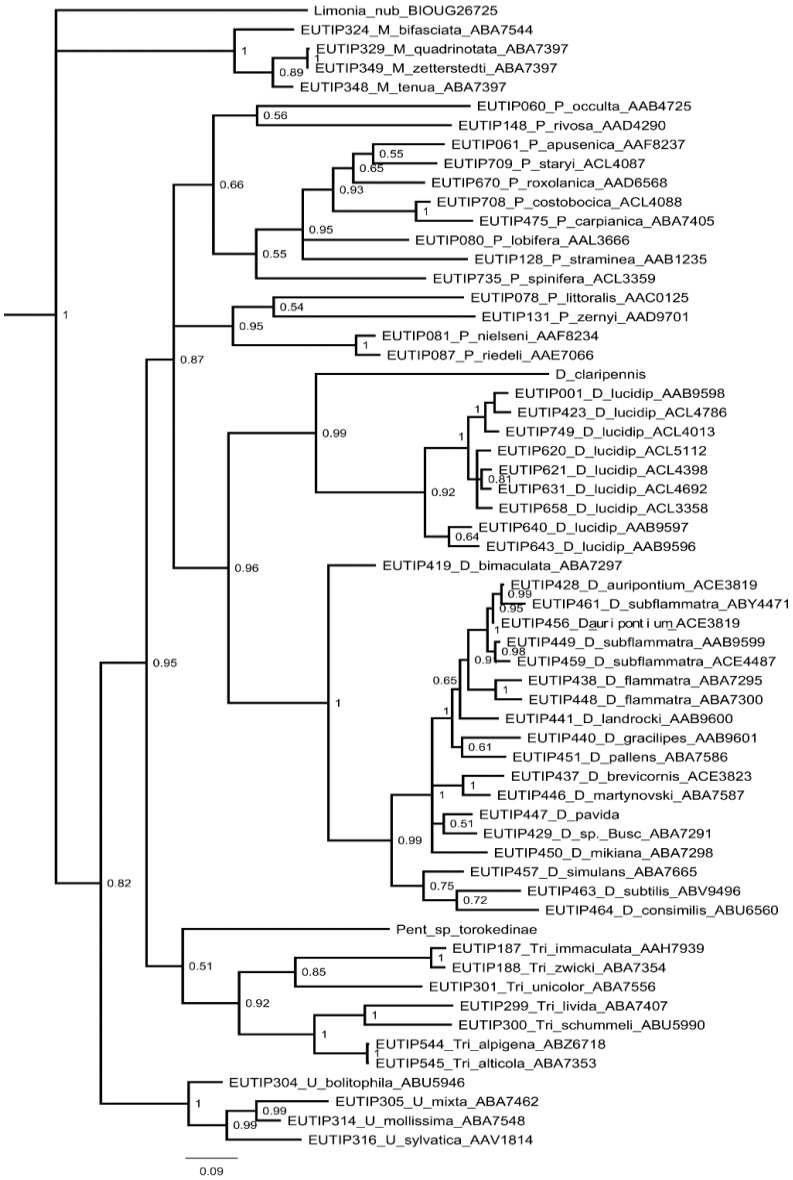

Fig. 3. BI species tree of *Dicranota* Zetterstedt, 1838, with the inclusion of the other *Pediciidae* species. Node values represent PP

3.2.Divergence within the *Paradicranota* subgenus, with the description of a new species

3.2.1. *Molecular genetic variability*

Both BI (Fig. 4) and ML trees show well defined and supported clades for all the species included in this study. Larvae sequences (marked as: D_sp_BIN) clustered together with the adults. BIN ABA7291 is a differentiated clade represented by morphologically undescribed individuals. This lineage clusters together with *Dicranota pavida* (Haliday, 1833), with a *p*-distance of 3.75% and a mean distance of 5.64%.

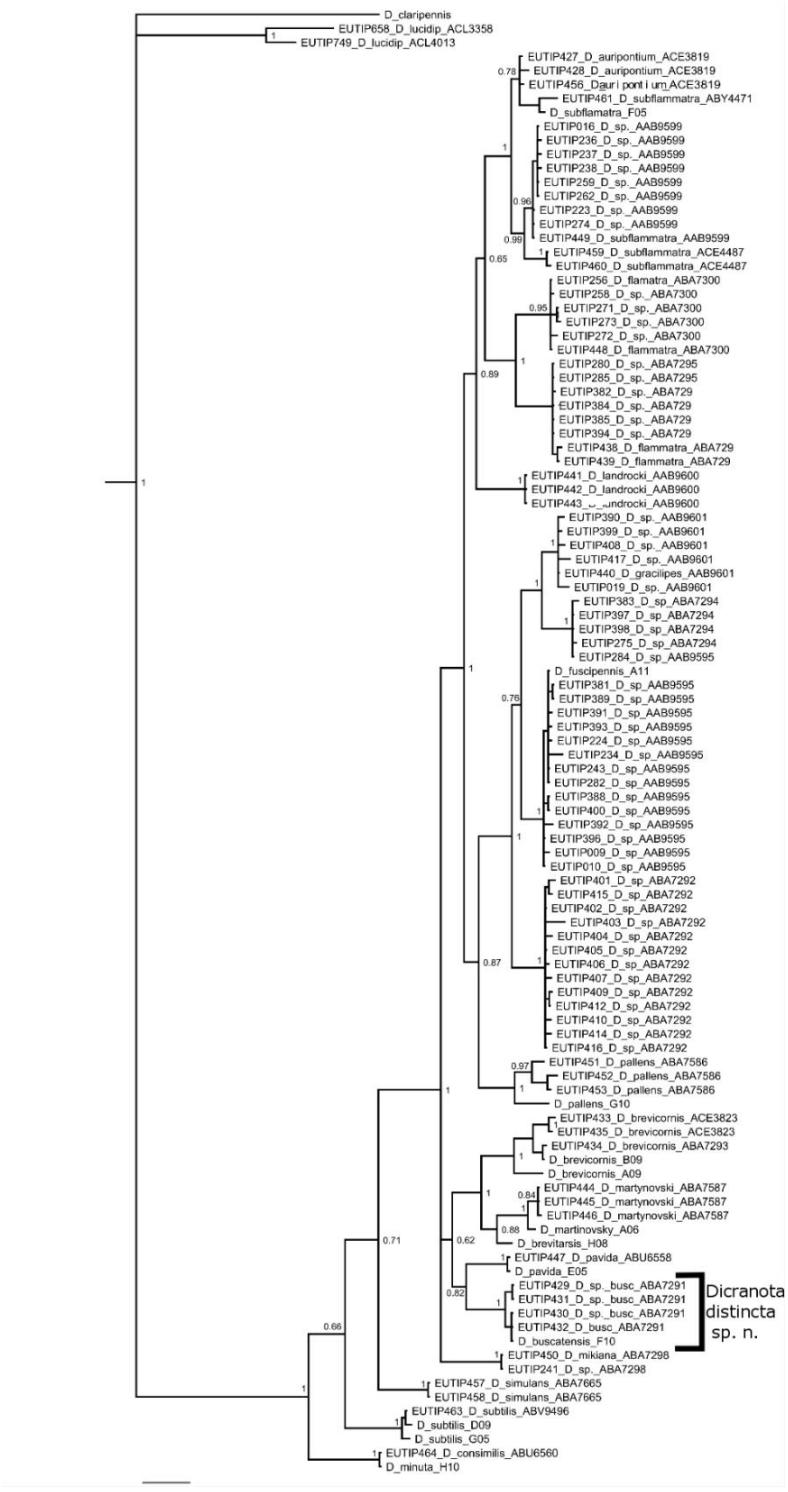

Fig. 4. BI species tree of *Paradicranota* species. Node values represent PP

3.2.2. *Morphological differentiation and the description of Dicranota (P.) distincta Keresztes and Kolcsár, new species*

The morphological analysis focused on the specimens representing the ABA7291 BIN, as these were the undescribed adult individuals that also showed distinct morphological characteristics. We give the discriminate characters of the new species in comparison with *D. pavida* based on the genetic results.

***Dicranota* (*P.*) *distincta* sp. n.**

BOLD accession number: EUTIP429 – EUTIP432; BIN: ABA7291

Material: Holotype male, four paratypes males: Romania, Stațiunea Muntele Băișorii, 1279 m, Gilău Mts., Buscat, 24 May, 2008, leg. Keresztes L.

Other material: 4 males, Romania, Romania, Stațiunea Muntele Băișorii, 1520 m, Gilău Mts., Buscat, 12 May, 2012, leg. Kolcsár L-P.; 1 males, 2 female, Șesuri, Rodnei Mts., Bistrița Aurie Valley, 17 May, 2014, leg. Keresztes L. and Kolcsár L-P.

Diagnosis: The new species belongs to the subgenus *Paradicranota* based on short antennae with 10 rounded flagellomeres and supranumerary crosswein present in r1 and pterostigma indistinct. The species is most proximal with *D. (P.) pavida* (Haliday, 1833), but with sharply distinct hypopygium, especially on the shape of the 9th tergit posterior margin and gonostylus lobes (Fig. 5-G).

Description: Male: Body uniformly dark brown, 5.5-6 mm. Head dark brown, almost black, with grey pruinosity. Antennae 12 segmented, scapus and pedicel light brown, flagellomeres darker. First flagellar segment elongate, almost twice as long as the second one. The following segments nearly spherical. Verticils sparse and short, almost as long as the length of the respective segment.

Thorax generally dark brown. Praescutum with three distinct, weakly shining, dark brown, longitudinal stripes and grey pruinosity between stripes. Scutum lobe with two large pale brown patch well separated in the middle. Pleurae light brown with grey pruinosity. Scutellum and postscutellum uniformly dark brown. Wings clear, with stigma weakly evidentiated (Fig. 5-D). Veins light brownish. Venation similar with the other members of the subgenus, Rs moderatelly long, slightly arcuated at the base, arising well beyond Sc2, in a distance that is longer than its own length and almost equal with the sectorof R2+3 between the two supernumerary cross-vein and R2. Discoidal cell absent. Halteres pale brown. Legs in type material all broken. Abdomen uniformly dark brown with dark grey pruinosity. Male terminalia (hypopygium) dark brown, gonocoxite and gonostylus lighter brown (Fig. 5-D). Posterior margin of the 9th tergit between ergal arms with two proeminent lobe separated with deep depression. Tergal arms comparatively long and stout, slightly curved, apex widened in a page-like shape.

Gonocoxite with a well developed apical lobe. Interbases well developed, superficially similar with the interbasis of *D. pavida*, but with a conspicous large hatchet-like cutting edge, arched dorso-laterally. Outer gonostylus (or outer dististyle) lobe-like, but oblong, sharply strangulate in the middle, and conical at tip, set up with strong sete in the distal half. Inner gonostylus (or inner dististyle) conspicuoulsy shaped (somewhat similar with *D. auripontium*), well developed, and strongly excised in the middle with a sharp oblique carina. Aedeagal complex

relatively large. Female: In general appearance resembling to males. Cerci massive, dark brown, slightly curved upward.

Etymology: The species epithet *distincta* translates to “highly different” and it was formed by the *Latin* adjective *distincta*.

Ecological notes and distribution: The species has a disjunct distribution in the Carpathians, in the Apuseni Mts. (Buscat) and the Eastern Carpathians (Rodnei Mts, Tarcău and Poiana Mărului), both well-known glacial refugia of the aquatic insects in the Carpathians, with high number of endemic species. The specimens belonging to this species were collected in the beginning of spring, right after snowmelt at high altitudes (between 1000 and 1500 m), along cold-stenotherm springs and headwaters.

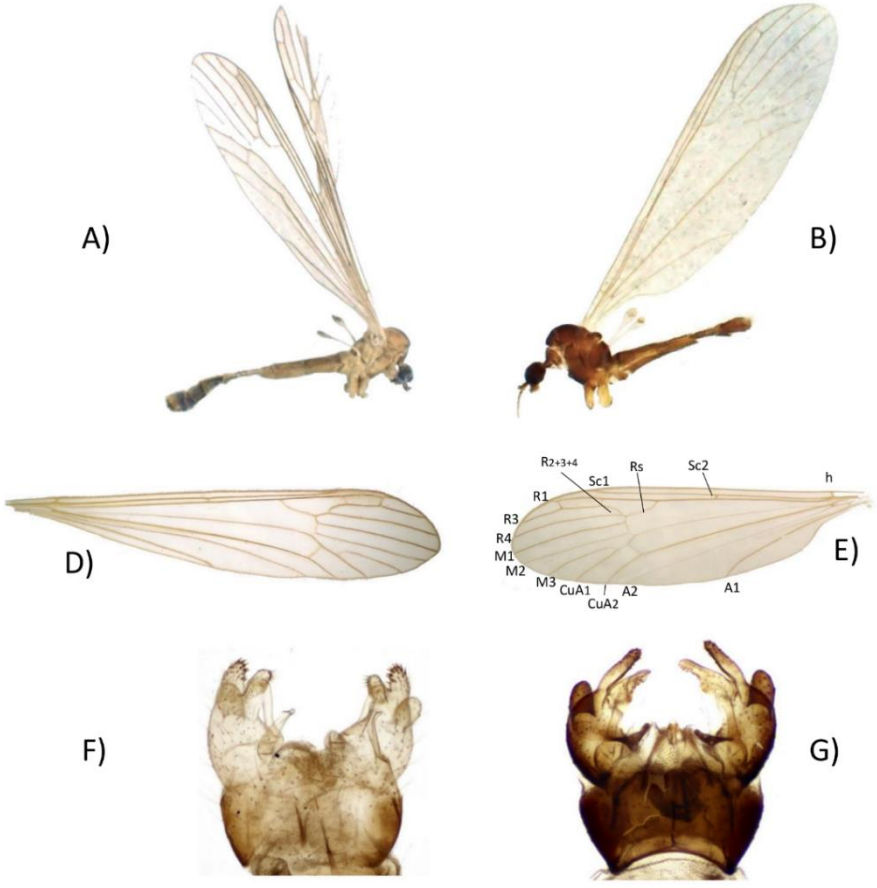

Fig. 5. Male adult, wing and hypopygium of *D. pavida* (A, D, F respectively), and of *D. distincta* (B,E,G respectively). Photo E also shows the names of the majour veins of the wing. Photos by Lujza Keresztes

3.3. Taxonomic revision of *Dicranota* (*Ludicia*) *lucidipennis* – Description of new *Dicranota* (*Ludicia*) species

3.3.1. *Molecular genetic variability*

The generated phylogenetic trees show eight well differentiated *D. lucidipennis* lineages, corresponding to the identified BINs. The basal clade is represented by AAB9596 and AAB9597 from the French, German, Swiss and Austrian Alps. They both represent well diverged lineages with a *p*-distance of 5.36%. This group acts as root to the clade containing two well separated clusters. One represented by two BINs from the Carpathian Mts and the Italian Alps, with a strong support (PP = 1, BP = 96%; pairwise *p*-distances of 2.76%). The second clade is represented by four BINs from the Balkan, that cluster together (PP = 0.71, BP = 0.50%; average *p*-distance is 2.57%). The differentiation of the eight lineages are also shown by the MJN (Fig. 6).

3.3.2. *Description of the differentiated taxonomic units*

The morphological analysis also showed clearly distinguishable characteristics between the observed mitochondrial.

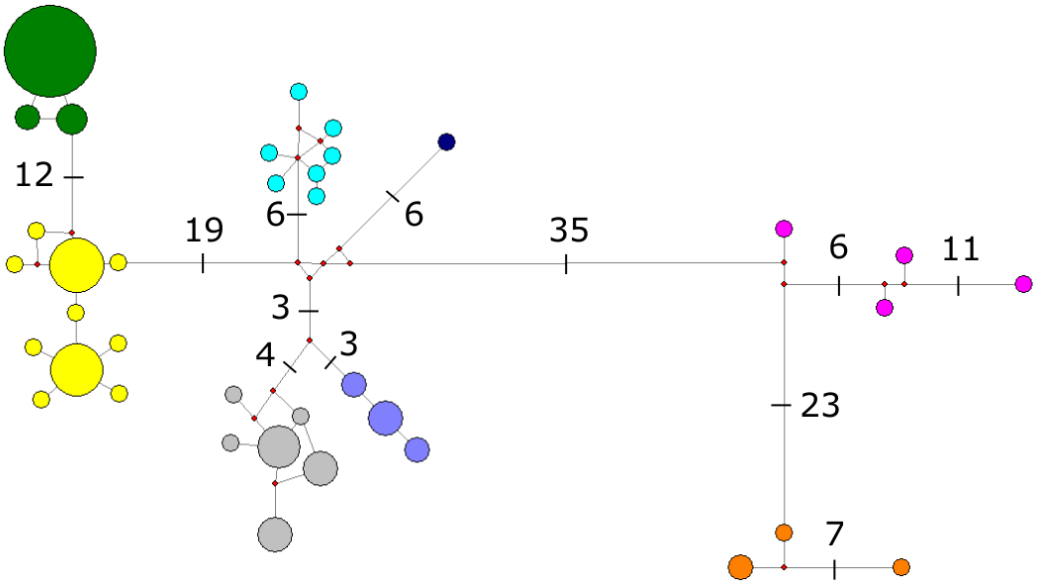

Fig. 6. MJN; circles represent the different haplotypes; numbers on the branches show the mutational steps between the haplotypes; colors represent the eight BINs: AAB9598 (yellow); ACL4786 (green); ACL5112 (light blue); ACL3358 (dark blue); ACL4692 (purple); ACL4398 (grey); AAB9596 (orange); AAB9597 (pink).

Redescription of *Dicranota* (*Ludicia*) *lucidipennis* (Edwards, 1921)

Material: AAB9598

Description: Head dark greyish with antennae almost as long as thorax, and having17 segments, blackish, second segment lighter. Thorax grey, praescutum with four slightly shining blackish stripes, the middle pair close together or partially fused, scutellum more-or less pale. Abdomen dark, the ventral side and hypopygium lighter, brownish-reddish or yellowish (Fig. 7-A). Wing almost clear, rather broad, anal angle well marked (Fig. 7-D).

On the distal half a conspicuoushairy field along veins is present (lacking in *D.* (*L.*) *claripennis*). Wing length 10-12 mm. Legs are brownish, with femur and tarsi lighter in its distal end. Hypopygium has the 9th tergit distal edge more or less straight, without a well-developed corner and a long-recurved process on each side. Gonocoxite has dorsal apical lobe with spinules. The knob-like tip of interbasis rounded and curved inward with an interior thorn oriented more-or less upward. The interbasis has a rounded curved end (Fig. 7-C). Outer gonostylus lobe-like, hairy with spinules, inner gonostylus digitiform (Fig. 7-B).

Ecological notes and distribution: The typical "*lucidipennis*" form is widely distributed in the Central and Western Part of Europe, and was also detected by us in France, Germany, Italy, Ukraine and Romania.

***Dicranota* (*Ludicia*) *luteipennis* (Alexander, 1975), stat. n.**

Pedicia (*Tricyphona*) *luteicolor* Alexander, 1975

*Material: Balkan clades;*BIN: ACL5112, ACL4692, ACL4398, ACL3358.

Description: General appearance resembling that of *D.* (*L.*) *lucidipennis*, but smaller and color of the body general light-brownish, 7-9 mm. Head yellowish grey, antennae 16 segmented, scape and pedicel light brown, flagellum dark brown ((Fig. 7-E). Prescutum light brown, with darker reddish-brownish middle stripes longer and fused, lateral stripes shorter, scutum yellowish. Wings narrow and shorter than of *D.* (*L.*) *lucidipennis*, generally light yellow, wing veins darker yellow (Fig. 7-H). Wing length 8-9 mm. Legs with coxae and trochanter lighter yellowish, femora darker yellow, tibia and tarsi gradually darkened to tip. Abdomen light brown, lighter in the middle. Male hypopygium darker, with tergite distal edge straight, the lateral outer corners not produced. Tergal arms long and slender. Gonocoxite with a well-developed apical lobe with black setae. The outer gonostylus lobe-like, with abundant black setae, inner gonostylus blade shaped, laterally flattened with very sparse small setae on the lower margin (Fig. 7-F). The knob-like tip of interbasis oval and curved inward with an interior thorn oriented downward (Fig. 7-G).

Dicranota* (*Ludicia*) *praedicta sp. n.

BIN: ACL4786.

Material: Holotype male, two paratype males.

Type locality: Romania, Păltiniș, Bătrâna and Rodnei Mts., 1657 m, 2014.06.01. leg. Keresztes L.

Diagnosis: Generally similar to *D.* (*L.*) *luteipennis* but differ by a general light-yellow color and details in hypopygium, especially in the shape of the interbasis, with tip ending with a prolonged rostrum oriented laterally.

Description: Male body length 8 mm. The darker head greatly contrast with a lighter yellowish body (Fig. 7-I). Head generally grayish, the anterior part of the vertex dark grey, the posterior part lighter grey, but with a dark stripe in the middle. Antennae with 16 segments. Scape dark brown, pedicel lighter brown, flagellum black. Thorax dorsal light yellow. Praescutum with three brown stripes, the middle stripe is longer and broader, the two lateral stripes are shorter, oval-like, close to the posterior part. Pleurae yellow. Wing 9 mm, yellowish, with dark yellow venation (Fig. 7-L). Legs with coxae and trochanter yellow, femora yellow in the proximal part, dark brown in the rest, as well as tibiae and tarsi. Abdomen yellowish orange, darker toward tip. Male hypopygium similar to those of *D.* (*L.*) *luteipennis* but differ on the shape of the interbasis. The interbasis has in dorso-interior position a dog-head shape tip (Fig. 7-K). Female: unknown.

Etymology: The species epithet *praedictus* translates to "prediction" and it was formed by the Latin adjective *praedictus*. The new species name is given by the prediction of its presence based on divergent morphological structures.

Ecological notes and distribution. The species is restricted in few remote enclaves in the Carpathians, like Rodna Mts. And Cibin Mts., both well-known glacial refugia in this mountain range, with high number of endemics among aquatic insects. The specimens belonging to this species were collected in the beginning of summer at high altitudes (up to 1000 m), along cold-stenotherm springs and headwaters.

Discussion. The species is close related to *D.* (*L.*) *lucidipennis* and *D.* (*L.*) *luteipennis*, having a lighter yellowish general body coloration, but differ from both by the presence of a dark stripe in the posterior part of the vertex and shape of the interbasis.

Dicranota* (*Ludicia*) *repentinus sp. n.

BIN: AAB 9596, AAB 9597.

Material: Holotype male, 1 paratype male.

Type locality: France, La Grande Croix, Vanoise National Park, Rhone-Alps, 1727 m, 2012.06.30. leg. M. Bálint.

Diagnosis: Generally similar to *D.* (*L.*) *lucidipennis* but differ in general light brownish color and details in hypopygium, especially in the shape of the interbasis with tip ending as an oval knob.

Description: Male body length 10 mm. Head generally black grayish. Antennae with 17 segments. Scape dark brown, pedicel brown, flagellum black. Thorax dorsal yellowish brown. Praescutum with four brown stripes, the middle stripes are fused more or less together, the two lateral stripes are shorter, triangle-like and fused together close to the posterior part. Pleurae lighter, yellowish. Wing 10 mm, transparent, with brownish venation. Legs with coxae and trochanter light brown, femora brown in the proximal part, dark brown in the rest, as well as tibiae and tarsi. Abdomen reddish-orange, last two segments dark brown. Male hypopygium yellow, similar to those of *D.* (*L.*) *lucidipennis*, but differ in the shape of the interbasis. The interbasis has in dorso-interior position a bird-head shape tip. Female: unknown.

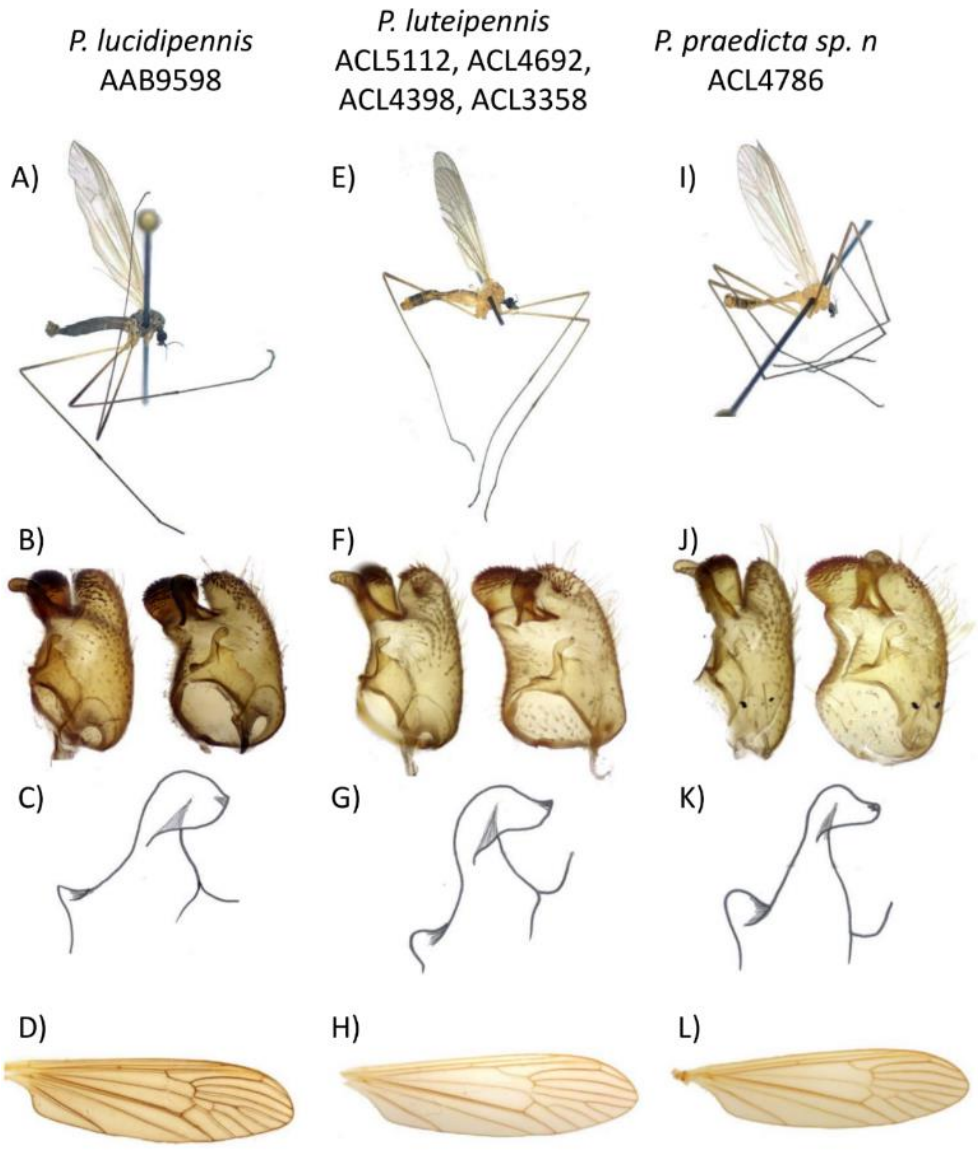

Fig. 7. *P lucidipennis*, *P luteipennis* and *P proedicta sp* n male adult (A, E, I), gonocoxite and gonostylus (B, F, J) interbasis drawing (C, G, K), wing (D, H, L). Photos and drawings by Lujza Keresztes.

Etymology: The species epithet *repentinus* translates to “unexpected” and it was formed by the Latin adjective *repentinus*. Morphologically very similar to the sibling *D. lucidipenis*, their deep genetic divergences were unexpected, this is the reason of the name of the new species.

Ecological notes and distribution. The species was collected in Vanoise National Park, in the Rhone-Alps at 1727 m above sea level, along small brooks, in early summer.

Discussion. The species is close related to *D.* (*L.*) *lucidipennis*, but with the general body color lighter, and differ on the shape of the interbasis.

4. Discussions

4.1. *Taxonomic position of the subgenus Ludicia Hutson and Vane-Wright, 1969 based on the mtCOI sequences*

This is the first study to examine the position of *Ludicia* with molecular methodes. Based on the *mt*COI barcode sequences, this subgenus clusters together with the other *Dicranota* subgenera with strong support (PP = 96), but it forms a well differentiated clade, but the clear differentiation, and the evident morphological distinctievness of these species suggest the need of a more thorough taxonomic revision with the inclusion of the other subgenera and maybe a multimarker approach.

4.2. *Cryptic diversity and the role of the Carpathians and the Balkans as a refugia and speciation center*

Dicranota (*L.*) *lucidipennis* show patterns similar to other aquatic and semi-aquatic groups, with large distribution (Schmitt & Varga, 2012). The presence of the species in the Apuseni Mts and the Eastern Carpathians suggest at least one potential refugia of this species somewhere in the northern part of the Transylvanian basin. The presence of this refugium is also confirmed by the presence of the newly described *Paradicranota* species, *Dicranota* (*P.*) *distincta* that is restricted to the Crapathians, with distribution in the Apuseni region and the Rodnei Mts.

Dicranota (*L.*) *praedicta sp. n.* is also endemic and is distributed in the Eastern Carpathians and in the eastern part of the Southern Carpathians. The low genetic distance and the morphological similarity between *D* (*L.*) *lucidipennis* and *D.* (*L.*) *praedicta sp. n.* suggest that the speciation event most likely occurred sometime during the Quaternary glaciation as observed between *Pedicia (Crunobia) costobocica* Kolcsár, Keresztes & Dénes 2016 and *Pedicia (Crunobia) carpianica* Kolcsár, Keresztes & Dénes 2016 (Dénes *et al.*, 2016a,b). All these species show patterns already observed in other Diptera (for example Ujvárosi & Bálint, 2012) and some caddisflies species (Pauls *et al.*, 2009) confirming the importance of the Carpathian mountain system as a refugia and speciation center for the aquatic and semi-aquatic diversity.

VI. Final conclusions

This thesis provides strong evidences on the importance of the Carpathian Mountains as one of Europes biodiversity hotspots. The morphological and molecular genetic work carried out in the frame of these studies led to the discovery and description of six new species out of which five are restricted to the Carpathians. All findings of this thesis confirm the role of the Carpathians as speciation center, but also, due to the long evolutionary histories shown by the studied groups, they highlight the cumulative character of the region as a refugium.

The *mt*COI barcode sequences showed a good resolution for our investigations. The taxonomic question regarding the position of the *Ludicia* subgenus was answered, placing it as a sister clade to all other *Dicranota* species.

The cumulative pattern of diversity and distribution of the range-restricted endemic species included in this thesis underline the importance of some mountain areas in the preservation of the present autochthonous aquatic diversity. Particular centers for diversification hosting several endemic species in the Carpathians are the northern Carpathians (Chornohora-Maramureș-Rodnei), the southern Carpathians (Bucegi Mountains), and the Apuseni Mountains. The present work supports the high conservation value of cold-stenotherm aquatic habitats in the Carpathians and emphasizes the conservational implication of the cryptic diversity. The future of these highly specialized range-restricted endemics depends on the proper management of these unique ecosystems in Europe.

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