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**Integrative taxonomy of the Romanian stoneflies (Insecta,
Plecoptera) and implication in biological assessment of
freshwaters based on molecular data**

SUMMARY

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SUMMARY

Stoneflies (Insecta: Plecoptera) are an ancient, hemimetabolous aquatic insect group, with egg, nymph and adult life stages, very essential in aquatic and terrestrial food webs, important in biological assessments, their evolutionary history, helps to understand biogeographical and phylogenetic processes and relations between different taxa, also they possess unique evolutionary traits. All these make them a really special, yet underexplored insect group, and raise the attention to the conservation of them.

Plecoptera taxonomy began in C. Linné's historical work, *Systema naturae* (1758), and up to the XXI. century, when an integrative taxonomical approach started to be applied, due to new available molecular methods, morphological characteristics, mainly genital structures of adults meant the basis of stonefly taxonomy, among some ecological and behavioral traits, such as drumming signals. Despite the large-scale availability of these new molecular methods, for Romania DNA sequences of stoneflies are scarce, practically non-existent in any of the international databases (BOLD, NCBI).

The work of Kis Béla is outstanding and primer in the taxonomy of Romanian Plecoptera, 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 a globally known reference work for the Carpathian stonefly fauna. The Carpathians, as major biodiversity hotspot for aquatic insects, due to the geographical position (with emphasize on isolation), lower altitude, fragmentation, heterogeneous geomorphology and landscape, well-conserved habitats, and low impact of Pleistocene glaciation, have always received special attention in the studies of Plecoptera fauna of Romania.

As a major aquatic insect order, stoneflies indicate the quality of freshwater habitats, by reacting fast to habitat degradation, changes in thermal conditions, and nutrient enrichment, however their larvae identification seems impossible at species-level. DNA-barcoding methods aim to ease the species-level identification of morphologically cryptic taxa and life stages, especially in the case of bioindicator insects, improving the bioassessment of aquatic habitats. This method is efficient only when comprehensive barcode reference libraries exist for certain taxa.

Outline of the thesis:

The first and main objective of this thesis is updating our knowledge about the Romanian stonefly fauna from an integrative taxonomical point of view, that includes molecular data, behavior traits and classical morphology. Up to present molecular data about the stonefly species

of the country are scarce, although these can mean key information nowadays, when genetical approach is becoming more popular and available, being applied not just in taxonomy, but also in bioassessment.

As the molecular marker used in our studies was the *mt*COI barcode sequence, the second important aim of this study is to test the efficacy of barcoding methods in the taxonomy of stoneflies, and highlight its significance in improving biomonitoring, where the species-level identity of morphologically cryptic larval stages is very important, yet impossible to achieve based on morphological characteristics in the case of Plecoptera, one of the most sensible bioindicator taxa.

By testing the efficacy of barcode sequences in the species-level identification of stonefly larvae, that are almost impossible to identify based on morphology, and additionally by creating a national stonefly barcode reference library, our third main objective is to provide useful molecular information and a new effective tool to all the Romanian biomonitoring systems and conservation managers. The application of barcoding methods will lead to a better conservation strategy of our freshwater habitats, that are highly threatened by biodiversity loss, environmental change, degradation, pollution and extinction.

As our study area includes mostly the headwaters of the Carpathian Mountains' Romanian parts, a major biodiversity hotspot in Europe, recognized as the EC10 Biogeographical region (Illies 1966), the fourth aim of this study is to underline the region's importance, in the context of stonefly biodiversity, as a hotspot of speciation and endemism.

These objectives are pursued in the chapters of the thesis as follows:

CHAPTER III: Faunistic data of Plecoptera from Romania – an annotated checklist of species based on recent data

The stonefly fauna of Romania is well documented in the Fauna Book of Romanian Plecoptera (Kis 1974), but since the publication of this basic literature, several new papers were published, that add new faunistic data to the country's list. Also, our stonefly collection contains new species to the fauna and new species to science, that supplement our knowledge about stoneflies in global and national level. As a result, we present in this chapter an annotated checklist of the stonefly species of Romania. Beside this, we focused on the Capniidae family, where major revisions were made in the last decade, that changed the systematic relations. According to this we provide a new identification key to the Romanian species belonging to this family. In this chapter we provide a detailed morphological description of a new *Zwicknia* Murányi, 2014 species, with

additional molecular, behavioral and ecological data of the species, according to an integrative taxonomical approach. We also indicate the existence of two new *Nemoura* Latreille, 1796 species, but here we provide just a brief presentation of the species and their morphology. According to the above mentioned content of the chapter, this sustains the first and forth aim of the thesis.

CHAPTER IV: Setting up a comprehensive DNA barcode library of Plecoptera from Romania – integrative taxonomy case studies based on adults

Nowadays molecular methods and especially DNA barcoding become a major basis of integrative taxonomy and bioassessment. The *mtCOI* barcode sequence seems to improve the taxonomy of different taxa, as well as stonefly taxonomy, by helping the detection of new species, cryptic species or genetical diversity within known species, and forms our general conception about species delimitation. This is possible due to the fact, that the barcode region, in most of the cases, has lower intraspecific variability than interspecific variability, making it a useful tool that helps the delimitation of species based on the barcoding gap between molecular sequences. This method is functioning well and can be applied in taxonomy, only if there are reliable reference barcode sequences from clearly defined, morphologically well-identified species. Databases that gather these molecular sequences are called barcode reference libraries. There are global, national and regional initiatives to create such reference libraries for stonefly species too, because they are not just useful in taxonomy, but also improve the biomonitoring of aquatic habitats, in the case of stoneflies, a major bioindicator group, where the species-level identification of larvae is rather challenging even for specialists. According to these, we initiated a Romanian stonefly barcode reference library, based on mostly sequences from adult specimens, to establish the basis of integrative taxonomy of Romanian stoneflies, and improve the national bioassessment of freshwater ecosystems. We started to build up this reference library from a biodiversity hotspot of aquatic insects, the Apuseni Mts., highlighting the need for further examination of these hotspot regions in the case of stoneflies. Therefore, this chapter tries to fulfill all the aims of this thesis.

CHAPTER V: Biological assessment of freshwaters using bioindicator taxa – a contribution on larvae data

The *mtCOI* barcode sequences are a proven tool in the species-level identification of morphologically cryptic life stages of aquatic insects, among them stonefly larvae, that are recognized as important bioindicator components of freshwater ecosystems, due to their sensibility of changes in aquatic environment. This means that we can obtain detailed, high-quality data of aquatic communities and their species composition, leading to a better understanding of the functioning and fragility of freshwater ecosystems. By applying barcoding methods, the species-

level identification of stonefly larvae can be achieved on a larger scale, being available also for non-experts. So, we can increase the amount and accuracy of data regarding stoneflies' ecology, role and significance in aquatic ecosystems, that might help the management and conservation of their habitats. This is achievable only if we have reference barcode sequences from well-identified, adult specimens of all the stonefly species present in a certain region, country. We started building up such a reference database for the Romanian stonefly species and tested the efficacy of barcoding in the species-level identification of stonefly larvae from well-preserved, natural freshwater habitats, the headwaters of Someșul Cald River, in the Apuseni Mts. Relying on our data national biomonitoring systems, freshwater managers and conservation decision makers, can get more accurate data about our aquatic ecosystems, therefore they can work out better conservation strategies for these habitats. Thus, this chapter is dedicated to sustain the second and third main objective of this thesis.

Keywords: mitochondrial DNA, barcode reference library, BOLD, checklist, species-level identification, Capniidae, *Zwicknia*, stonefly larvae, Carpathians, biodiversity hotspot, identification key, bioassessment

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GENERAL CONCLUSIONS

- (1) This thesis includes important morphological, behavioral and molecular data about stoneflies. By adding new species to the Romanian stonefly fauna and discovering three new species to science, it supplements our general knowledge about stoneflies on a national and global level, and supports the application of integrative taxonomic approach in Plecoptera research.
- (2) The updated checklist of Romanian stoneflies provides from now on a basic reference for further taxonomic and ecologic studies, and facilitates national bioassessment of freshwater habitats. According to the new stonefly checklist presented in our study, now there are 120 species, belonging to 23 genera and 7 families.
- (3) As a result of our work there are available DNA barcode data of stoneflies from Romanian populations in international molecular databases (BOLD), that can be applied in general stonefly taxonomy.
- (4) We initiated for the first time a DNA barcode reference library of Romanian stoneflies, that supports the integrative taxonomy of stoneflies and the application of molecular methods in local bioassessment of flowing freshwater habitats. We aim to continue and develop this reference database with high-quality and well curated molecular data.
- (5) We proved that DNA barcode sequences can improve the species-level identification of cryptic stonefly larvae, that are important bioindicator organisms of aquatic ecosystems and help water quality bioassessments. We suggest the application of these available and effective new methods in official national bioassessment protocols.
- (6) Our study provides useful molecular data that can be implemented in more specific bioassessment protocols of freshwater habitats, providing detailed and specific data about aquatic communities, helping specialist to achieve a better understanding of freshwater ecosystems and conservation managers to take more accurate actions to preserve aquatic biodiversity.
- (7) This thesis provides strong evidences on the importance of the Carpathians as major biodiversity hotspot of aquatic insects, especially stoneflies. We suggest further studies of mountainous regions to discover a more realistic dimension of stonefly biodiversity from the Romanian Carpathians and Apuseni Mountains.