

Review of :

Diversity and speciation of mayflies (Ephemeroptera) from the Caucasus and adjacent areas

By Ľuboš Hrivniak

I have read with great interest the present dissertation. The core of the thesis is based on six papers, five of them being already published and the sixth is under review. Nevertheless, the whole thesis remains homogeneous and well written. Five articles were published in suitable peer review journals with the best possible impact factors according to subject of the paper: namely, three papers in *Zootaxa* and one paper in *Zookeys* which are the most common journals for alpha-taxonomic papers with an IF around 1. The last paper was published in the revue with high reputation and strong quality requirement: *Molecular Phylogenetics and Evolution*. With the choice of these journals, Ľuboš Hrivniak insures a validation of the quality of the results, an international visibility of his researches and a recognition of his expertise for the topics.

I am very impressed by the multitude of skills shown by Ľuboš Hrivniak: field sampling, identification of the specimens, morphological descriptions, drawings and pictures, molecular lab and analysis. I am going to details these different skills.

I never had the opportunity to be in the field in Caucasus, but after some fieldtrips in Arabian Peninsula, Israel, Levant, Africa and others, I can easily imagine the pleasure but also the difficulties of collecting mayflies in this area. I have the habit to read the alpha-taxonomic papers of the supervisor, Pavel Sroka and of the consultant for this thesis Roman Hodunko (Godunko). I can easily recognize their fingerprints and that both of them trained Ľuboš Hrivniak and taught their best way to draw with precision and efficiency. All the important characters are well illustrated, ventral and dorsal view are given, variation of shape and setation are illustrated (variations between specimens or between earlier and later stages). The authors also often added high quality colour pictures which is of great importance for the colouration of the tergites and sternites. They completed the illustrations with SEM pictures which provide more precise details of some structures often difficult to observe with standard optical material. All in all, I can only underline the extreme quality and accuracy of the different kind of illustrations. Amazingly, there is almost no difference of quality between the first and the last papers, proving the expertise of the author for this kind of work and also the accurate supervision offered by the senior authors.

For the first alpha-taxonomic papers, Ľuboš Hrivniak sequenced CO1, the very commonly used mitochondrial gene, often considered as the barcode. Of course, CO1 by itself is not sufficient to separate the lineages in unambiguous species. Moreover, using mitochondrial gene present several advantages but also disadvantages. However, Ľuboš Hrivniak was aware of these limitations; he always used CO1 correctly and for the right purpose. CO1 is perfect for delimitation of evolving lineages representing species hypotheses. Ľuboš Hrivniak never considered CO1 to reconstruct higher phylogeny (which must be avoided because of rapid saturation, and also because the history and evolution of a single mitochondrial gene does not necessary reflect the species history). The author carefully combined morphological and molecular characters to obtain total evidence and high support for his species hypothesis. Lab protocol and molecular analysis follow conventional methods but remain accurate and highly tested. Intra- and inter- specific distances were calculated (with K2P distances). Trees reconstruction present probably the most important differences between the first paper and the following: at first one, only NJ (Neighbour Joining) and ML (Maximum Likelihood) methods were used, then more complex, powerful and reliable Bayesian methods were added. The protocol used for species delimitation also evolved between the papers. First, Ľuboš Hrivniak

only used ABGD (Automatic Barcode Gap Discovery) for assigning organisms into hypothetical species. He then also delimited the independent clusters with the GMYC model (General Mixed Yule Coalescent model). For Iran fauna, the individual species were supported by morphology as well as by ABGD and GMYC methods.

While acting as a reviewer for the manuscript on the *Caucasiron* from Iran, I pointed out that the distinction of the different species of *Caucasiron* remains a great challenge. I considered that, without a dichotomic key, species identification is almost impossible. Rather than just adding a short key at the end of the paper, the authors submitted a new paper with a complete identification guide for the fifteen species of *Caucasiron*. They also provided for each species the main diagnostic characters, a distribution with a map and the main ecological traits.

With his paper on the diversification of *Caucasiron* during the Miocene, Ľuboš Hrivniak opened interesting perspectives; he experimented new techniques and acquired new skills. He used multiple genes approaches and therefore obtained more supports to the basal nodes. Interestingly, he can prove that *Caucasiron* present more affinities with Asia than Central Europe. He also demonstrated the importance played in the speciation process by the orogeny of the Caucasus mountains rather than the climatic oscillations of the Quaternary; this result was rather unexpected as these oscillations are considered as the driver of speciation in Central Europe for several groups. This contribution is also important for the understanding of the phylogeny of the genus *Epeorus*, in particular for the validity of the different subgenera and the evolution some important morphological characters such as the suction disc. For me, it was also a great opportunity to learn more about the orogeny and land development of the Caucasus.

During this review, I have almost not said a word on the parts one and three of the thesis. The part one is a global introduction to the Caucasus, the mayflies in general and the genus *Epeorus* and subgenus *Caucasiron* in particular. This introduction is especially important for those who still haven't read LH's publications and for none specialist of mayflies. This part is generally well-written and facilitate the read the following chapters. I remain less enthusiastic by the last chapter. The perspective and future challenges of the present researches seem rather limited. I would have expected more originality and a little beat of "wind of madness"! Or it was maybe made on purpose to give to the reviewers matter for questions?

In conclusion, where most other authors limited their contributions to molecular reconstructions with species hypotheses or to description of new species based on morphological characters, Ľuboš Hrivniak provided morphological and molecular evidences to support his new species and to properly separate already described ones. The challenge was even greater when we look at the way the previous species were described: generally based on limited amount of material and only one stage (larvae or imagos), with the description and illustration regularly incomplete or inaccurate. By adding a complete key of *Caucasiron* and ID card for each species, he did not only achieve his taxonomic work, but he made his research accessible and useful for a large majority of hydrobiologists (and not only to a very restricted set of specialists). This is probably the best valorisation we can expect. The chapter on the diversification of *Caucasiron* in the Caucasus is certainly the master piece of his thesis as demonstrated with its publication a relevant journal.

Therefore, I can only conclude that I highly recommend the acceptance of the present thesis without any restriction.

Questions:

- 1) If we try to compare the subgenus *Caucasiron* in the Caucasus and *Epeorus s.s.* in Central Europe, in the Alps in particular, we can remark that both taxa are very successful. However, these successes are quite different: why the speciation process results of so many species in the Caucasus and only two in the Alps? (Ecological niches? Species competition? geological events? Gen flows?). Can we imagine that if we apply the methods in the Alps than in Caucasus, we discover that in fact we are also in front of two complexes of cryptic species?
- 2) The aim of the chapter III is to give a tool for the routine identification of *Caucasiron* to non-specialists. Should it be an important point for hydrobiologists to give some kind of Red List or at least to identify the most threatened species? and what are the main factors responsible for local extinction or rarefaction?
- 3) All the species of *Caucasiron* are endemics to Caucasus (including sometimes surrounding areas) while, based on the results from Armenia, other lineages present wide West Palearctic distribution. Can we postulate that the taxa from middle to high altitudes are endemic to Caucasus and are more related to Central Asia taxa while species from lower altitudes are more widely distributed and present affinities with Central Europe? Or what is just an artefact due to the lack of relevant studies at low altitudes species?
- 4) Finally, a remark rather than a question: the title indicates “diversity and speciation of **mayflies** from the Caucasus”... than part I section 5, the author states that *Caucasiron* is **the best** model for evolutionary study. Maybe, I should remind to the author that mayflies encompass other genera and other families and to be sure that *Causasiron* is the best model, other models should be tested (*Rhithrogena* or even some Baetidae)



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Assessment of Ľuboš Hrivniak's Ph.D. thesis

**Diversity and speciation of mayflies (Ephemeroptera)
from the Caucasus and adjacent areas**

The Ph.D. thesis submitted by Ľuboš Hrivniak is a comprehensive study of the diversity and evolutionary history of mayflies (Ephemeroptera) from the Caucasus. The overall aim of the thesis is to provide complex information about diversity, distribution, identification, and evolutionary history of the mayflies from the Caucasus mountains. More specific goals of the thesis are to document mayfly diversity in the Caucasus Mountains, to revise the taxonomy and systematic position of *Caucasiron*, and to clarify the origin and factors driving the evolutionary diversification of Caucasian mayflies, using a multilocus phylogenetic and biogeographical analyses of the model subgenus *Caucasiron*.

The thesis is divided into three parts. The Part One provides general information about mayflies, their worldwide distribution and diversity, the speciation and phylogeography of mountain aquatic insects, geological and climatic history of the Caucasus, the current state of knowledge of mayflies (Ephemeroptera) of the Caucasus and information on systematics and biogeography of *Epeorus* (*Caucasiron*) as the model group for the evolutionary study. The overall aims of the thesis and specific aims for following chapters are clearly defined at the end of this part. In this part, the author has proved excellent knowledge of literature and very good understanding of each scientific problem he studied. Moreover, he writes very clearly and engagingly.

Part Two contains five published articles and one manuscript, divided into four chapters. The first chapter includes one article dealing with the diversity and distribution of mayflies from Armenia and description of a new species *Ecdyonurus eurycephalus*. The second one consists of three articles describing five new *Epeorus* (*Caucasiron*) species from Georgia, Turkey, and Iran. High quality drawings and photos supplement detailed morphological descriptions. Delimitation of new species is verified by the analyses of COI (barcode) sequences of all Caucasian species of the subgenus *Caucasiron*. Besides that, the distribution and habitat preferences of *Epeorus* (*Caucasiron*) species in the study region are assessed. Chapter III is a manuscript of an identification guide to larvae of Caucasian *Epeorus* (*Caucasiron*). The authors provide an identification key and describe and illustrate the diagnostic characters of all species, including previously unknown larva of *Epeorus* (*Caucasiron*) *insularis*. In addition to high-quality illustrations and photographs, the article provides data on the distribution and ecological preferences of studied species. The study is of great practical importance because it enables the routine identification of *Caucasiron* larvae necessary for biomonitoring and hydrobiological research in the Caucasus region. In Chapter IV, consisting of a comprehensive article published in a highly impacted journal, the author met the goal to clarify the origin and factors driving the evolutionary diversification of Caucasian mayflies. Additionally, he elucidated the phylogenetic relationships in the genus *Epeorus*, which has significant implications for its systematics.

Part Three provides a concise summary of the results, and future perspectives in the research of Caucasian mayflies.

The core of the presented Ph.D. thesis consists, with one exception, of published articles that have undergone a rigorous review process. The rest of the thesis is very clear, logically and engagingly written. I did not find any reason to criticize, therefore I only have a few questions for the author to discuss.

1. Is it realistic to expect that several unknown *Epeorus* species will be found in Europe when examining the genus in more detail using integrative taxonomy methods?

2. I see obvious parallels in the geological history of the Caucasus and the Carpathians; for example, some parts were islands in the Miocene. Do you expect that it could be reflected in the formerly undiscovered diversity of mayflies in this area, like what was shown for the genus *Gammarus*?

I consider the Ľuboš Hrivniak's Ph.D. thesis to be of exceptional quality. The thesis met the objectives, brought original results in the field of taxonomy, systematics, and biogeography of mayflies, and therefore it can be accepted without hesitation.

Bratislava, August 16, 2020



Tomáš Derka