**‘’BABEȘ-BOLYAI” UNIVERSITY FROM CLUJ-NAPOCA**

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# Integrative taxonomy of the Romanian aquatic and semiaquatic true flies (Insecta, Diptera) and implication in biological assessment of freshwaters based on molecular data

SUMMARY

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**SUMMARY**

Diptera, or true flies, represent one of four megadiverse insect groups: Diptera, Coleoptera, Lepidoptera, and Hymenoptera, which make up the majority of Earth's animal biodiversity. The evolutionary origins of Diptera trace back to the Late Permian–Early Triassic period (approximately 245 million years ago), with fossil and phylogenetic evidence suggesting their divergence from ancestral Mecopteran lineages, alongside the orders Trichoptera, Lepidoptera, and Siphonaptera (Hennig 1981, Krzemiński and Evenhuis 2000, Marshall 2012). With such a long evolutionary history behind them, dipterans have successfully colonized all biogeographic regions and ecological zones, including extreme environments such as Antarctica (Usher and Edwards 1984). Dipterans constitute an important part of the biodiversity of aquatic and semiaquatic environments, as well, with endless adaptations to a large variety of water types (Adler and Courtney 2019).

The name Diptera is derived from the Greek words *di* (two) and *ptera* (wings), referring to the defining morphological feature of the group: the presence of a single pair of functional wings. The second (posterior) pair of wings is evolutionarily reduced to a small structures known as halteres. This adaptation to flight with a single wing pair has resulted in the pronounced development of the mesothorax, the middle thoracic segment, which serves as a key morphological character in the identification of many dipteran species (Yeates and Wiegmann 2005).

Diptera undergo complete metamorphosis (holometabolous), characterized by a life cycle comprising four distinct stages: egg, larva, pupa, and adult. The egg stage is typically short, lasting from a few days to several weeks. Larval development generally involves three instars in Brachycera and four in lower Diptera, although certain groups such as Simuliidae, Tabanidae, Thaumaleidae, and some Chironomidae may exhibit additional instars. The duration of the larval stage can range from approximately two weeks to several months, depending on the taxon and environmental conditions. The pupal stage also varies in duration across species. Adult longevity is highly variable, ranging from less than two hours in some Deuterophlebiidae to several weeks or even months in other taxa (Courtney et al. 2017, Adler and Courtney 2019, Sarwar 2020).

The order Diptera is traditionally divided into two suborders, the suborder Nematocera- these are ‘primitive’ flies, a heterogeneous assemblage of generally delicate flies with multi-segmented antennae (such as black flies, crane flies, mosquitoes, moth flies, non-biting midges, fungus gnats) and the suborder Brachycera which are ‘advanced’ flies, generally more compact flies, with shorter, stylate antennae (house flies, horse flies, bee flies, snipe flies). Both groups are divided into a number of superfamilies and families (Oosterbroek 2006, Marshall 2012).

Nematocera is rather a artificial, paraphyletic group, with some members more closely related to the suborder Brachycera and are characterized by primitive or default (plesiomorphic) characteristics similar to those found in closely related orders (like Mecoptera). Brachycera is a natural, monophyletic group, with characteristics not shared with other groups. The suborder Brachycera can be divided in 'lower Brachycera' and Empidoidea on a side and a higher Brachycera (or Cyclorrhapha) on the other hand (Yeates et al. 2007, Marshall 2012).

**Aquatic and semiaquatic Diptera, a brief summary**

In aquatic ecosystems, Diptera represent more than half of all recorded insect species (Sundermann et al. 2007), occupying an exceptionally wide range of habitats– including seas, oceans, shoreline saline pools, all kinds of stagnant waters (e.g., lakes, ponds, and marshes), groundwater zones, cold or hot springs (Courtney and Cranston 2015). They are able to survive and thrive all freshwater habitats and often dominate the benthic macroinvertebrate community in terms of abundance (Sundermann et al. 2007, Marshall 2012, Dobson 2013, Oosterbroek 2016, Borkent et al. 2018). Most dipteran larvae are free-living and actively move within their habitats, which can include water, sediments, saturated wood, wet leaf packs, fruit, or decaying organic matter. While families such as Chaoboridae, Chironomidae, Culicidae, and Simuliidae are typically aquatic, others exploit sediments (Ceratopogonidae, Psychodidae, Tabanidae, Tipulidae), wood and leaf packs (Axymyiidae, Limoniidae, Syrphidae), or decomposing organic substrates (Ephydridae, Muscidae, Sarcophagidae, Sphaeroceridae), illustrating the wide ecological diversity of larval habitats within Diptera (Courtney et al. 2017, Adler and Courtney 2019). In general, it is difficult to delimit aquatic species from those 'water dependent' (Wagner et al. 2008, Dobson 2013). The pupal stage of aquatic Diptera also reflects this ecological diversity and can be classified into four major categories: (1) free-swimming pupae that rise to the surface at adult emergence (e.g., Chaoboridae, Culicidae and most Chironomidae); (2) pupae that are attached to benthic substrates (e.g., Blephariceridae, Deuterophlebiidae, Simuliidae and some Chironomidae, Psychodidae and Tipuloidea); (3) pupae that burrow into marginal substrates (e.g., Athericidae, Tabanidae, and some Tipuloidea); and (4) pupariation (e.g., Ephydridae, Sciomyzidae, Stratiomyidae and Syrphidae), which is a special form of pupation within the integument of the last larval instar (Adler and Courtney 2019).

While most adult aquatic Diptera remain near water, some species disperse several kilometers from aquatic habitats to obtain blood meals required for ovarian development (Ceratopogonidae, Culicidae, Simuliidae, Tabanidae), whereas others spend much of their adult life skimming the water surface (Chironomidae, Empididae, Dolichopodidae). Overall, most adults have only limited direct association with aquatic environments (Adler and Courtney 2019).

According to recent estimates, approximately 46,000 Diptera species undergo at least one developmental stage associated with the aquatic environment, either as larvae or pupae (Adler and Courtney 2019). Globally, out of the 158 recognized Diptera families, 41 families (representing about 26%) include species with aquatic stages in their life cycle. Among these, 20 families are considered aquatic at least during the larval stage (Adler and Courtney 2019). In Europe, a total of 130 dipteran families have been recorded, of which approximately 25 are associated with aquatic habitats (Oosterbroek 2006).

## Biological assessment of freshwaters using aquatic and semiaquatic Diptera, problems and perspectives

Aquatic Diptera are excellent indicators of water quality (Adler and Courtney 2019). Knowledge and understanding of aquatic life are essential for understanding of natural history, evolution, and ecology.

The first documented biomonitoring assessment dates back to 1907, when the saprobic index was used to detect organic pollution in Central Europe (Kolkwitz et al. 1909). Since then the methods have evolved and diversified using different biological groups.

In order to protect aquatic ecosystems, it is important that pollutants, regardless of their nature, can be detected and monitored. A primary objective of freshwater biomonitoring is to assess the relative impacts of pollution on the living communities within surface waters (Metcalfe 1989, Morse et al. 2007). Over the past decades, aquatic ecosystem assessment and biomonitoring programs have been implemented globally to support Environmental Impact Assessments and to provide robust data for the protection and restoration of freshwater ecosystems (EU Water Framework Directive, US Clean Water Act) (Elbrecht and Leese 2017a). Macroinvertebrates have been recognized as key indicators of the ecological integrity of freshwater systems and are currently the most widely used organisms in water quality assessments worldwide (Leese et al. 2018, Ge et al. 2021). Aquatic insects represent particularly robust bioindicators of freshwater quality, owing to the fact that many taxa display distinct and often predictable responses to variations in environmental conditions, thereby providing valuable insights into ecosystem health and anthropogenic impacts (Metcalfe 1989, Lenat 1993, Barbour et al. 1999, Morse et al. 2007, Sundermann et al. 2007, Elbrecht and Leese 2017b, Adler and Courtney 2019, Santos and Ferreira 2020).

The implementation of the Water Framework Directive (WFD) in 2000 ensured that the integrity of ecosystems became a central consideration in the management of water resources (EC 2000). The main aim of the WFD is to establish a comprehensive framework for the implementation of sustainable water management strategies, ensuring the long-term protection of water resources. Member States are required to assess the ecological status of their water bodies by comparing current conditions with the expected reference state, using a rigorously structured and standardized species-level monitoring system (Leese et al. 2016, 2018). The primary goals include the protection and improvement of the quality of the aquatic environment and the achievement of “good status” for all surface and groundwater bodies by 2015 (EC 2000) or by 2007 at the latest (Poikane et al. 2014).

A multihabitat sampling method is used, enabling a more taxon-specific assessment of river conditions across key bioindicator groups (Cheshmedjiev et al. 2011). This methodology incorporates seven principal indices: saprobic, Ephemeroptera-Plecoptera-Trichoptera (EPT), Oligochaeta-Chironomidae, family richness, Shannon-Wiener diversity, feeding type, and stream zonation preference (AQEM 2002). The calculation of these indices, which collectively generate the Multimetric Index (MI) used to characterize the ecological status of freshwater habitats, requires species-level identification and comprehensive knowledge of the bioindicator traits associated with each species.

Identifying individuals at the species level using morphological criteria in most groups remains a significant problem (if not impossible), especially in the case of morphologically cryptic larvae. From the depths of the oceans to the springs on mountaintops, aquatic life characterizes environmental conditions and provides services to a wide range of communities (Rouillard et al. 2018, Borgwardt et al. 2019). For these reasons, the capacity to evaluate aquatic biodiversity and track its temporal changes is of paramount importance, not only for preventing biodiversity loss but also for safeguarding human well-being (Weigand et al. 2019).

**Aim of the thesis**

(1) The main objective of this doctoral thesis is to update and expand the current knowledge of the Romanian Diptera fauna through an integrative taxonomic approach that combines molecular data, classical morphological analysis, and ecological characterization, including trophic roles and habitat preferences. This research aims to fill critical knowledge gaps by generating and applying genetic information. The integration of such data is becoming increasingly vital, not only for accurate species delimitation in taxonomy, but also for its applications in ecological assessment and biomonitoring practices.

(2) The second aim of this research is to evaluate the utility of barcoding methods, specifically *mt*COI barcode sequences, for species identification within Diptera taxonomy.

Species-level identification is very important, yet classical morphological approaches often fail to distinguish cryptic species.

(3) The third main objective of this study is to improve national bioassessment protocols for freshwater ecosystems with new bioindicator species suitable for biomonitoring in Romania. This will be achieved through the development of a comprehensive genetic reference database (DNA barcode library) for local Diptera taxa.

## MATERIALS AND METHODS

The sampling sites include freshwaters from the Carpathians, designated as the EC10 Biogeographical region by Illies (1966), as the majority of regional (endemic) or overlooked Diptera fauna are located in different mountainous waters.
Samples were collected from the Eastern Carpathians— expecially Rodna, Maramureş and Bucovina Mountains— the Southern Carpathians, including the Bucegi, Făgăraş, Parâng, and Retezat-Godeanu Mountains, and the Western Romanian Carpathians, specifically the Banat and Apuseni Mountains. A total number of 371 different sites were investigated between 2018 and 2024 (Figure 3). Detailed descriptions are provided for sites where species newly recorded for the country were found. Several specimens collected by other people were also included and examined in this thesis, the names of the collectors were noted where applicable.

## RESULTS

Between 2018 and 2024, over 20,000 Diptera specimens were collected from various sites (Figure 3). From these, a total of 8,832 aquatic and semiaquatic Diptera individuals (4,366 larvae, 387 pupae, 3,267 adult males, and 812 adult females), representing 32 families (18 families within the suborder Nematocera and 14 families within the suborder Brachycera) were selected for morphological analysis (Appendix 1).

### Reevaluate biodiversity of aquatic and semiaquatic Diptera in Romania, based on morphology and molecular data – taxonomic revision of selected groups

DNA barcode sequences were successfully obtained from a total of 1,643 specimens, comprising 614 larvae, 21 pupae, and 1,008 adults. A total of 465 distinct barcode sequences corresponded to 323 species. Among these, 99 barcode sequences are new contributions to the BOLD database. Overall, 587 aquatic and semiaquatic Diptera species have been documented in Romania, with 63 species reported for the first time in the country (Appendix 1).

**Morphology and molecular data reveal the presence of *Mochlonyx* Loew, 1844 in the Carpathians with an annotated list of Chaoboridae (Insecta, Diptera) from Romania**

Phantom midges (Chaoboridae, Diptera) are flies that belong to a family of aquatic lower Diptera (Culicomorpha) which are closely related to mosquitoes (Culicidae) (Sæther 1970, 1997, Borkent 2012, da Silva et al. 2020, Lorenz et al. 2021). Adults are highly similar to Culicidae in the wing venation, presence of scale-lake setae on the wing margin, and large plumose antennae in males, but phantom midges have smaller mouthparts and the females do not blood feed (Borkent 2012, Salmela et al. 2021).

Larvae and pupae of phantom midges are aquatic and found frequently in different types of permanent or ephemeral standing waters (Sardella and Carter 1983). Larvae develop through four instars. The full-grown, last instar larvae are small (6–9 mm) to large (10–22 mm) (Sæther 2002, Salmela et al. 2021) with strong mandibles and prehensile antennae that they use to grasp their prey, especially micro crustacean and Culicidae larvae (Lock et al. 2014, Kruppert et al. 2019). The presence of two or three pairs of air sacs serves to adjust larvae position in water according to their predatory behaviour (Teraguchi 1975). The large majority of phantom midge larvae live in smaller ponds, frequently in ephemeral ponds, which are formed by melted snow or heavy rains in early summer, and they have a system of metapneustic spiracles that are adapted for aerial respiration (*Mochlonys* and *Cryophila*). *Chaoborus*, the only truly lacustrine genus, has developed an apneustic system and cutaneous respiration (Krogh 1911, Cook 1956). *Chaoborus flavicans* Meigen, 1830 and *Ch. crystallinus* (De Geer, 1776) are the only exceptions that are closely related to larger lakes with fishes, due to their vertical migration and adaptation to low oxygen content of greater depths, thereby avoiding predation by fish (Dawidowicz et al. 1990). Pupae have conspicuous articulated swimming paddles on the last abdominal segment, and a specific respiratory system that helps pupae to regulate an up-right position in waters and stability during emergence (Parma 1971, Borkent 2012).

Only 53 species of Chaoboridae have been described globally (Salmela et al. 2021). According to the latest classification of Chaoboridae (Sæther 2002), the family is represented by only 6 extant genera worldwide. With the exception of the cosmopolitan genus *Chaoborus* A.A.H.Lichtenstein, 1800, all other genera have a more restricted distribution. Representatives of *Mochlonyx* Loew, 1844 are Holarctic, the species of *Cryophila* Edwards, 1930 are Palearctic, *Eucorethra* Coquillet, 1903 is Nearctic, *Australomochlonyx* Freeman, 1962 and *Promochlonyx* Edwards, 1930 belongs to the Australian region. The 11 different European Chaoboridae species belong to three genera, *Cryophila* Edwards, *Mochlonyx* Loew, and *Chaoborus* Lichtenstein

(Edwards 1930, Borkent 1993, 2011, 2014, Salmela et al. 2021), from which only 6 species were recorded from Central Europe so far (Sæther 2002).

## *Twinnia hydroides* Novák, 1954 (Diptera: Simuliidae) in the Romanian Carpathians: integrative molecular and morphological data shed light on a long-standing dilemma

Species-level identification has become the cornerstone of bioassessment of freshwaters in recent decades (Sayer et al. 2025). The widespread availability of high-resolution morphological studies and molecular tools enables a thorough survey of biodiversity even in taxonomically difficult or neglected taxa (Ratnasingham and Hebert 2007, Stein et al. 2014, Weigand et al. 2019). Simuliidae (Insecta, Diptera) make an important contribution to aquatic ecosystem services due to the filter-feeding habits of larvae in different types of freshwaters (Ciadamirado et al. 2016, Tepliuk 2019). Adults have major medical and veterinary importance, as their blood-feeding females can be vectors of pathogens in many parts of the world, including Europe (Rivera and Currie 2009, Adler et al. 2010, Hamada et al. 2010, Adler and McCreadie 2019).

*Twinnia* Stone and Jamnback, 1955 is a small genus of Simuliidae, with only 10 species in the Holarctic area (Adler 2025). The genus was first described by Stone and Jamnback (1955) in North America and was named in honour of C.R. Twin, who made outstanding contributions to the knowledge of Canadian black flies (Simuliidae). *Twinnia* is separated from the closest related genus *Gymnopais* Stone, 1949 by several morphological characteristics in adults, pupae, and larvae (Stone and Jamnback 1955, Jedlička and Stloukalová 1997).

The majority of *Twinnia* species are distributed in Asia (six species) and North America (three species) (Adler 2025). In Europe, only *Twinnia hydroides* (Novák 1956) has been recorded (Usova 1987, Jedlička and Stloukalová 2004, Jedlička 2006). However, problems and uncertain data surrounding the taxonomy and distribution of *T. hydroides* have remained. This can mostly be traced to the difficulty of scientific communication, typical of the 1950s, among researchers in different parts of the world, as well as the lack of up-to-date regional faunistic data, which still questions the distribution of the species in the Southeastern Carpathians (Kúdela and Stloukalová 2007, Stloukalová and Jedlička 2007, Jedlička 2019; Adler 2025).

Genus *Twinnia* (as *Gymnopais*) was reported for the first time from the Palearctic area by Novák (1956), describing the species *T. hydroides* as *Gymnopais* sp. (*hydroides mihi*), now placed in the genus *Twinnia* (Jedlička 2019). The original description was based on one female pupa and one pupal exuvia from the Lower Tatra Mts. (Lučianka below Ďumbier, Slovakia), following the taxonomy and nomenclature of Rubtsov (1940). The name of the species "*hydroides*" was based on the pupal gill filaments resembling a hydra (Cnidaria) (Novák 1956).

However, the description of *T. hydroides* presents a challenging taxonomic issue, recently summarized by Jedlička (2019) in his excellent synthesis of Novák's life and research. The taxonomic description can be reconstructed from numerous sources and indirect evidence, though it sometimes involves unexpected developments and questionable records. In the same year as the description of *T. hydroides*, Rubtsov published his monograph on the Simuliidae fauna of the (former) Soviet Union (Rubtsov 1956), listing the species as *G. hydroides* Novák, 1956, accompanied by a detailed description but without citing any references. This description was likely based on a handwritten draft provided by Novák, supplemented by additional material (male, male pupal exuvia and larvae) collected and reared in the laboratory by Novák himself, which he sent to Rubtsov for examination (Novák 1957a, Jedlička 2019). Subsequently, Novák revised the genus designation to *Twinnia* (following the work of Stone and Jamnback 1955) and redescribed the species in 1957 as *Twinnia hydroides* sp. n., using newly collected specimens from headwaters and mountainous habitats in Northeastern Bohemia (Czech Republic) and the High Tatra (Slovakia). This new material included larvae, pupae and adults, as well as additional collecting sites and morphological information (Novák 1957a, 1957b, Jedlička 2019).

When comparing *Twinnia* populations from the Carpathians (Slovakia) and Hercynian Mts. systems (Czech Republic), Novák noticed some minor differences in the morphology of larvae, males and females (but not pupae), as well as in the elevation and timing of emergence of different developmental stages. These observations lead him to describe a new species, *T. tatrensis* Novák, 1959, based on material from the Tatra Mts., collected from the same locality where *T. hydroides* was originally described, using the same pupa, exuvia and possible females that he previously used in the original description of *T. hydroides* (Novák 1956, 1957a, 1959, Jedlička 2019). Comparing more material from both species and observing overlapping morphological variability, Knoz (1980) later stated that the same species can be found in the Carpathians and the Hercynian Mts., namely *T. hydroides*.

The final twist in the story comes from the collection of the Zoological Institute, St. Petersburg (Russia), specifically from Pávay's collection, where a male specimen of *T. hydroides* was deposited and labelled with collection data from 1923 from the Rodna Mts., Romania (Yankovsky 2010). The male specimen of *T. hydroides* from the Rodna Mts., deposited in St. Petersburg, was most likely unknown to Novák (1956, 1957a, 1959) and Dinulescu (1966), who, in his comprehensive monograph on the Simuliidae of Romania, mentioned both species as potentially present in the Carpathians. Nevertheless, Dinulescu's data were treated as actual faunistic information by later authors (perhaps due to language difficulties in translating the original information published in Romanian). Information on faunistic data and distribution of *Twinnia* in Europe was later revised, mentioning the species from Slovakia, Austria, Czech

Republic, Germany, Italy, Poland, Switzerland, and Ukraine, but not from Romania (Rubtsov and Yankovsky 1988, Crosskey 1990, Jedlička and Stloukalová 2004, Stloukalová and Jedlička 2007, Adler 2025).

The main objective of our work was to contribute new data on the distribution of *T. hydroides* from the Southeastern Carpathians, and confirm the presence of the species in two distinct enclaves in the Apuseni and the Rodna Mts., Romania. Based on integrative data, we also want to solve the dilemma of whether one or two different Twinnia species occur in the Romanian Carpathians.

**Annotated list of aquatic and semiaquatic Diptera from Romania – contribution with new faunistic and taxonomy data**

A total of 32 Diptera families, comprising approximately 2,075 aquatic and semiaquatic species, have been previously recorded in Romania. Dipterans are the most species rich macro-invertebrate group in aquatic ecosystems investigated by us, with 587 species belonging to 32 different families, supported also by our DNA barcode data (Figure 80 and Appendix 1), of which 63 represent new records for the Romanian fauna. These newly recorded species are distributed among 37 genera across 17 families (Figure 81). This increases the known total number of aquatic and semiaquatic Diptera species in Romania to 2,138.

The DNA barcode library developed in this study constitutes the first regional benchmark dataset, encompassing 323 morphospecies, and has the potential to significantly enhance high-quality freshwater biomonitoring (Appendix 1). However, the substantial gaps identified within the regional DNA barcode library, together with the absence of comprehensive taxonomic revisions, continue to constrain the broader applicability of the mitochondrial cytochrome c oxidase subunit 1 (*mt*COI) marker for an efficient, economical, and reliable approach to species-level identification.

**Improving national bioassessment protocols for freshwaters based on molecular data of aquatic and semiaquatic Diptera species from Romania**

Approximately 2,075 aquatic and semiaquatic species of Diptera, belonging to 32 families, have previously been recorded in Romania. A total of 8,832 aquatic and semiaquatic Diptera individuals (4,366 larvae, 387 pupae, 3,267 adult males, and 812 adult females) representing 32 families were selected for morphological analysis. Our investigation identified 587 aquatic and semiaquatic Diptera species, including 63 new records for the national fauna.

These newly recorded taxa span 37 genera across 17 families, raising the total known number of aquatic and semiaquatic Diptera in Romania to 2,138 species.

Molecular data further enriched this inventory. DNA barcodes were successfully obtained for 1,643 specimens (614 larvae, 21 pupae, and 1,008 adults), yielding 465 unique barcode sequences corresponding to 323 species. Among these, 99 barcodes represent new entries in the Barcode of Life Data System, while an additional 46 sequences—although currently lacking species-level identification—also constitute novel contributions to the global reference library (Figure 83).

To facilitate the application of our results in biomonitoring, we provide a dataset of three essential bioindicator parameters—saprobic values, feeding types, and stream zonation preferences (Appendix 1, Figures 76–78). These parameters are required for the calculation of the multi-parameter index, the final endpoint of the multi-habitat sampling method applied in the specific monitoring of ecological status (Marcoci 1984, Schmidt-Kloiber and Hering 2015). For each species, the associated BINs are also provided through the BOLD system.

By generating new genetic data for 323 aquatic Diptera species and filling critical gaps in international reference databases this study contributes substantially to the improvement of biomonitoring practices. At the national level, the updated Diptera checklist, together with the integration of bioindication parameters, allows for the supplementation of the official list of bioindicator macroinvertebrates with 366 additional well-documented species.

## GENERAL CONCLUSIONS

(1) This thesis provides a comprehensive contribution to the knowledge of Romanian Diptera by integrating morphological, ecological, and molecular data. A total of 587 aquatic and semiaquatic Diptera species were identified, including 63 species newly recorded for the Romanian fauna. These newly documented taxa span 37 genera and 17 families, increasing the total number of known aquatic and semiaquatic Diptera species in Romania to 2,138. Feeding type and stream zonation preference are also provided for the identified species, along with the saprobic index where available.

(2) The results confirm the utility of DNA barcoding, specifically *mt*COI sequences, as an effective and reliable tool for species identification among aquatic and semiaquatic Diptera. DNA barcodes were successfully obtained from 1,643 specimens, resulting in 465 unique sequences. For 323 species, a high degree of congruence was observed between morphological data and molecular species delimitation, demonstrating the reliability of DNA barcoding when high-quality reference databases are available. We recommend the integration of these new,

accessible, and effective methods into official national bioassessment protocols, with the aim of improving the rigor and accuracy of ecological evaluations.

(3) A total of 46 new BINs correspond to specimens that could not be identified morphologically to the species level. These limitations reflect the developmental stage of the material (larvae, pupae, or female adults lacking diagnostic characters), the origin of the specimens from poorly investigated regions, and their affiliation with taxonomic groups that remain insufficiently studied in Romania (e.g., Ceratopogonidae, Chironomidae, Psychodidae, and Empididae). Moreover, some of these BINs likely represent previously undescribed species, highlighting the need for comprehensive taxonomic revisions, which we intend to address in future studies. The absence of corresponding reference sequences in international databases currently prevents species-level identification even using molecular approaches, revealing gaps in existing genetic repositories and underscoring the need for continued integrative taxonomic work.

(4) As a major outcome of this research, a comprehensive DNA barcode reference library was established, currently comprising 323 morphologically confirmed Diptera species from Romania. In addition, the identification of 366 newly proposed bioindicator species not only enriches the national faunal inventory but also offers valuable data that will support and improve the accuracy and effectiveness of freshwater bioassessment protocols. The 99 newly generated DNA barcode sequences represent a valuable contribution to international genetic databases, supporting efforts to enhance global biodiversity reference databases. Most of these novel records originate from communities of aquatic and semiaquatic Diptera that have been historically understudied, particularly those inhabiting overlooked high-altitude ecosystems, including rheocrene springs, spring-fed brooks, boggy springs, and mineral springs.

(5) Overall, this research advances the understanding of aquatic and semiaquatic Diptera biodiversity in Romanian freshwater ecosystems. By delivering high-accuracy identification tools, expanding the DNA barcode reference library, and highlighting overlooked taxa and habitats, the thesis provides a solid foundation for future taxonomic research, strengthens regional bioassessment protocols, and supports the long-term conservation and sustainable management of freshwater ecosystems.

## Scientific performance

### List of publications included in the thesis

**Terec AB**, Dénes AL, Dénes A, Jancsó B-Z, Dénes A, Keresztes L (2025) *Twinnia hydroides* Novák, 1956 (Diptera: Simuliidae) in the Romanian Carpathians: integrative molecular and morphological data shed light on a long-standing dilemma. The European Zoological Journal 92(1): 863–875. https://doi.org/10.1080/24750263.2025.2534163.

**Terec AB**, Dénes AL, Dénes A, Jancsó B-Z, Keresztes L (2024) Morphology and molecular data reveal the presence of *Mochlonyx* Loew, 1844 in the Carpathians with an annotated list of Chaoboridae (Insecta, Diptera) from Romania. North-Western Journal of Zoology 20 (2): 99–108. Article No.: e241303.

Keresztes L, **Terec AB**, Jancsó B-Z, Dénes AL, Dénes A (2023) Small flies with high conservation value: first reliable record of *Hyperoscelis veternosa* (Diptera, Canthyloscelidae) from Romania supported by DNA barcode data. Travaux du Muséum National d'Histoire Naturelle "Grigore Antipa" 66(1): 165–171. https://doi.org/10.3897/travaux.66.e105107.

Keresztes L, Cîmpean M, Battes K, Denes A, **Terec AB** (2021) *Dicranomyia (Idiopyga) nigristigma* Nielsen, 1919 (Diptera, Limoniidae) recorded for the first time from calcareous springs in Romania. Entomologica Romanica 25: 31–34. http://dx.doi.org/10.24193/entomolrom.25.4.

### List of publications not included in the thesis

Mabrouki Y, **Terec AB**, Taybi FA, Dénes A, Keresztes L (2023) Taxonomic notes and key to the West Palearctic *Antocha* (*Antocha*) Osten Sacken, 1860 (Diptera, Limoniidae) with description of a new species from Morocco. Biodiversity Data Journal 11: e103849. https://doi.org/10.3897/BDJ.11.e103849.