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Metabarcoding for identification of indigenous water fleas (Crustacea: Cladocera) and earlier detecting of the non-indigenous taxa: a gap analysis

Метабаркодирование для идентификации местных видов ветвистоусых ракообразных (Crustacea: Cladocera) и раннего обнаружения чужеродных таксонов: анализ проблем

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ABSTRACT. The next stage in the ecological monitoring of indigenous and non-indigenous species has been opened by the metabarcoding, laying the foundation of the species detection based on the DNA analysis directly in an environmental sample from a water body. Metabarcoding can be very helpful in the comprehensive assessment of biodiversity and the monitoring of harmful species, since it significantly reduces requirements to taxonomic skills and experience, allows fast performing of large-scale analyses, and is sensitive to specimens barely accessible to morphological identification such as juveniles of some species. The aim of this mini-review is to make a critical analysis of the "state of the art" of the cladoceran identification in most recent publications, where metabarcoding techniques were used, paying particular attention to its problems and practical difficulties. We are sure that after some years the eDNA methods will form a basis for the monitoring of indigenous and non-indigenous aquatic taxa, and such methods will be officially recommended by the environmental authorities of different countries. However, now we need to resolve the main problems concerning their use. If the problems of the low quality of the database entries, like misidentifications of taxa or presence of pseudogenes disguised as proper vouchers, etc. will not be resolved, many researchers will be misguided and many automated pipelines will give noisy or outright wrong results. We believe that current efforts of the cladocerologists need to be focused on the filling of the GenBank with sequences of all the loci widely used in the metabarcoding from all the known cladoceran genera and species in different regions of the world. Such efforts could be coordinated with the complete mitogenome (and full genome) sequencing initiatives which allows forming the basis for future eDNA studies, including, but not limited to the metabarcoding.

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РЕЗЮМЕ. Новая страница в экологическом мониторинге аборигенных и неаборигенных видов была открыта после использования метабаркодинга, заложившего основу для выявления видов на основе анализа ДНК окружающей среды (природная ДНК, экологическая ДНК, эДНК) из водоёма. Метабаркодинг может быть очень полезным для комплексной оценки биоразнообразия и мониторинга «вредоносных» видов, поскольку он значительно снижает требования к таксономическим навыкам и опыту исследователя, позволяет быстро проводить крупномасштабные исследования и идентифицировать образцы, трудные для морфологической идентификации, такие как ювенильные особи или фрагменты тел гидробионтов. Цель данного мини-обзора — критически проанализировать состояние исследований в области идентификации кладоцер по последним публикациям, в которых использовались методы метабаркодирования, уделив особое внимание проблемам и практическим трудностям. Мы абсолютно уверены, что через несколько лет методы эДНК станут основой для мониторинга аборигенных и неаборигенных таксонов гидробионтов, и такие методы будут официально рекомендованы природоохранными органами

разных стран. Однако, если не будут разрешены проблемы, связанные с ошибочными идентификациями таксонов в международных базах данных и с наличием в них последовательностей псевдогенов, замаскированных под надлежащие ваучеры, и т.д., многие исследователи будут введены в заблуждение, а автоматизированные системы будут давать «грязные» или откровенно неверные результаты. Мы считаем, что в настоящее время усилия кладоцерологов должны быть направлены на пополнение базы данных NCBI GenBank последовательностями локусов, широко используемых в метабаркодинге всех известных родов и видов, в частности ветвистоусых ракообразных, в разных регионах мира. Такие усилия могут быть скоординированы с работами по полному секвенированию митогеномов (и полных геномов), что позволит сформировать основу для будущих исследований эДНК, включая таковые, но не ограниченные рамками метабаркодинга.

Introduction

Microscopic crustaceans, including water fleas (Crustacea: Cladocera), are key links in the food chains in the continental aquatic ecosystems. It is well-known that the "traditional" methods of their identification (based on dichotomous keys and referring to morphological characters, comparison with species descriptions and figures in key-books) require intensive work of well-trained (during many years!) experts. It is obvious that such a routine taxon identification is laborious and time-consuming, and therefore it creates difficulties in its application for extensive monitoring and other ecological studies employing large sample sets. Moreover, using of regional keys could lead to omission of recently appeared non-indigenous taxa, habitually similar with their indigenous congeners, as the formers are absent in such keys [Kotov *et al.*, 2022]. Unfortunately, there were many examples of situations when invasive taxa were not detected by local scientists at earlier stages of their penetration to new regions, even in Europe with well-studied cladoceran fauna. For example, invasive status of *Daphnia ambigua* Scourfield, 1947 and *D. parvula* Fordyce, 1901 in Europe was confirmed many years after the Second World War, when they were occasionally transported from North America by military amphibious vehicles [Flössner, Kraus, 1976].

Application of molecular methods is regarded as a panacea for correct estimation of biological diversity, accurate species identification in ecological monitoring and earlier detection of non-indigenous taxa among cladocerans as well as among any other hydrobionts (see Hebert *et al.* [2003a,b]; although we will not discuss in this review non-cladoceran taxa). Indeed, application of molecular methods improved a lot the taxonomy of several cladoceran groups [Petrusek *et al.*, 2008; Adamowicz *et al.*, 2009], helped to resolve phylogeny of many macrotaxa [Van Damme *et al.*, 2007; Cornetti *et al.*, 2019; Neretina *et al.*, 2021], including the cladoceran orders [Xu *et al.*, 2021], and gave a phylogeographic explanation of recent global and local distribution patterns [Taylor *et al.*, 1998; Crease *et al.*, 2012; Kotov, Taylor, 2019; Zuykova *et al.*, 2019; Kotov *et al.*, 2021; Pereboev *et al.*, 2024].

Most genetic studies are focused on different groups inside the *Daphnia* O.F. Müller, 1785 genus [Petrusek *et al.*, 2008; Adamowicz *et al.*, 2009; Zuykova *et al.*, 2019; Pereboev *et al.*, 2025] which can be regarded as the most studied taxon of the invertebrates in continental water bodies. Applying of genetic methods led to revealing of cryptic invasions, fully missed by morphologists like in the case of the "American *pulex*" expansion to Africa and then to Mediterranean European countries [Mergeay *et al.*, 2005; Conde-Porcuna *et al.*, 2021; Vecchioni *et al.*, 2021]. Some earlier genetic works, unfortunately, have contributed to the increase of the *Daphnia* taxonomy uncertainty, but the situation has been greatly improved in the 21st century [Kotov, 2015]. Nowadays, it is common to accompany taxonomic revisions by phylogenies based on several genes and even full genomes [Kotov *et al.*, 2021; Pereboev *et al.*, 2025].

The next stage in the ecological monitoring of indigenous and non-indigenous species has been opened by the metabarcoding [Pompanon *et al.*, 2011], laying the foundation of the species detection based on the DNA analysis directly in an environmental sample from a water body [Ficetola *et al.*, 2008]. Metabarcoding can be very helpful in the comprehensive assessment of biodiversity and the monitoring of harmful species, since it significantly reduces requirements to taxonomic skills and experience, allows fast performing of large-scale analyses, and is sensitive to specimens barely accessible to morphological identification such as juveniles of some species [Milian-Garcia *et al.*, 2023; Nynatten *et al.*, 2023]. To date good examples of invasive species detection using environmental DNA (or eDNA) were provided [Brown *et al.*, 2016]. Recently the DNA metabarcoding has been started to be used also for analysis of the plankton community composition from archived samples fixed in formalin [Shiozaki *et al*., 2021]. Furthermore, a new direction was established: the study of the environmental RNA, which could lead to a "revolution in ecological resolution" [Yates *et al*., 2021]. At the same time, studies of the environmental DNA from sediments (sedDNA) have been started [Willerslev *et al.*, 2003; Tsugeki *et al.*, 2022]. The eDNA analysis (including metabarcoding) was already applied many times for the cladoceran identification in different continental water bodies [Yang *et al.*, 2017; Yang, Zhang, 2020; Valdez-Moreno *et al.*, 2021; Liang *et al.*, 2024] and seas [Stefanni *et al.*, 2018; Zamora-Terol *et al.*, 2020; Song, Liang, 2023].

However, even the authors of cited publications pointed out that there are still many problems concerning the application of metabarcoding [Cristescu, Hebert, 2018]. The aim of this mini-review is to make a critical analysis of the "state of the art" of the cladoceran identification in most recent publications, where metabarcoding techniques were used, paying particular attention to its problems and practical difficulties.

In this paper our main focus is metabarcoding based on short-read NGS platforms (e.g. Illumina MiSeq, etc.), however it should be acknowledged that the approaches

employing long-read ones (PacBio SMRT, Oxford Nanopore) have been developed recently and tested in zooplankton research [Moutinho *et al*., 2024]. For example, PacBio SMRT (Single-Molecule Real-Time) has been successfully applied in marine zooplankton metabarcoding [Lee *et al*., 2022] and even in high-scale barcoding of arthropods [Hebert *et al*., 2018], although it is mainly used for studies on microorganisms, e.g. fungi and protists [Tedersoo, Anslan, 2019; Jamy *et al*., 2020]. Unfortunately, the methods of long-read metabarcoding are not applying for the cladoceran studies, although there are no fundamental barriers to such works. The main advantage of the long-read metabarcoding is ability to obtain longer sequences (theoretically, up to many thousands, but usually barcodes no more than few kilobases are used) and, therefore, increased accuracy. Working with PCR products, high error rate of these platforms can be overcome due to sequencing of numerous copies of amplicons and bioinformatic processing of the obtained data [Hebert *et al*., 2018].

Difficulties of the eDNA metabarcoding

Methodological problems. Apparently, the success of the eDNA identification of a given species depends on its population density [Walsh *et al.*, 2019], ability to shed the exoskeleton [Trimbos *et al.*, 2021], body volume, and so on. The methods using eDNA are still developing now, and many methodological problems have not been fully resolved yet. For example, despite conclusions made by some recent authors [Bourque *et al.*, 2023], biotic and abiotic factors influencing temporal variation in the eDNA concentration are poorly known, as well as the speed of eDNA degradation [Seymour *et al.*, 2018].

In some publications a strong correlation was demonstrated between sequence abundances and microscopical individual counts [Song, Liang, 2023]. Nonetheless, the metabarcoding studies frequently result in a higher biodiversity as compared to traditional morphological identification [Schroeder *et al.*, 2020]. However, it ought to be inquired whether such differences could be explained by a real advantage of the metabarcoding method, or it is due to detection of occasional eDNA from adjacent biotopes, in-lab contamination, PCR artefacts, or insufficient training of the team in morphological methods?

The claim that eDNA is stable enough to preserve identifiable genetic fragments for prolonged time spans is partly withdrawn, although it still could be justified in some cases. On the other hand, we have to acknowledge recent progress in the genetic-based monitoring of aquatic environments due to use of eRNA instead of eDNA for the taxon detection. It is important that eRNA is not as stable as eDNA, and studying the former, we obtain a snapshot of biodiversity during a relatively short period of time before sample collection [Cristescu, 2019; Ankley *et al.*, 2022]. Nevertheless, the question about the coherence of the morphological and molecular methods is open for discussion.

Scarce information on any cladoceran species in international genetic databases. Paul D.N. Hebert was awarded the Benjamin Franklin Medal in Earth and Environmental Science just for the idea of the DNA barcoding [Petraitis, 2024]. The latter approach is a predecessor of metabarcoding, and the intimate link between them is obvious if we acknowledge that reference databases used for the latter are composed mainly from results of the former. In their review of eDNA studies, Cristescu & Hebert [2018: 212] specially noted that "a reference sequence library derived from the analysis of voucher specimens belonging to the taxonomic group under study is critical to support such work". Unfortunately, the cladoceran reference sequence library is still very incomplete, and no special efforts to create one have been made since their publication.

Nearly a million cladoceran sequences could be found in the Nucleotide database of NCBI GenBank but such a "good representation" is only an illusion. Actually, a great disproportion is observed between the number of sequences of *Daphnia* and other cladocerans: among 952011 sequences in the Genbank retrieved as "Cladocera [organism]" on 27.11.2024 the vast majority belongs to *D. magna* and *D. pulex* (Table 1). This is a very common situation in genetics, physiology, and ecology of the Cladocera: only *Daphnia* is relatively well-studied, while most other cladocerans are almost untouched by investigations [Smirnov, 2017; Portinho *et al.*, 2024].

Table 1. Results of the searches in the Nucleotide database of NCBI GenBank according to different keywords on 27.11.2024.
Таблица 1. Результаты поиска в базе данных нуклеотидов NCBI GenBank по различным ключевым словам на 27.11.2024 г.

Keyword	Total number of sequences retrieved by "Keyword[organism]"	Number of COI sequences retrieved by "Keyword[organism] AND (COI OR COX1)"
Cladocera	952011	8189
genus *Daphnia*	926788	2144
in part. *Daphnia magna*	601651	781
in part. *Daphnia pulex*	200675	129
Anomopoda	945564	5645
Ctenopoda	3236	1764
Onychopoda	2107	568
Haplopoda	1105	211

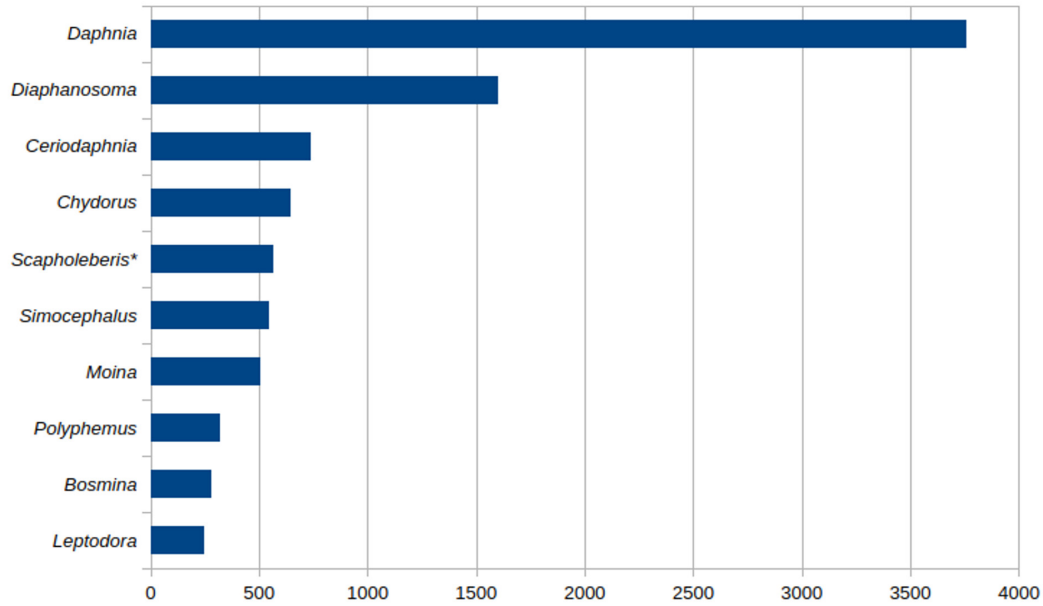

Fig. 1. Top 10 cladoceran genera by total number of marker gene sequences in the Nucleotide database of GenBank. For each genus the number is obtained by adding all the counts of "*genus*[organism] AND *term*" where the term is "(COI OR COX1)" or "12S" or "16S" or "18S". For *Scapholeberis*, the 12S sequences were not taken into account, since almost all of them were counted in the 16S ones due to the predominant locus being spanned across the two genes.

Рис. 1. Топ-10 родов кладоцер по общему числу последовательностей маркерных генов в базе данных нуклеотидов GenBank. Для каждого рода число получено путем сложения всех результатов по запросу "*genus*[organism] AND *term*", где *term* — "(COI OR COX1)", "12S", "16S" или "18S". Для рода *Scapholeberis*, последовательности гена 12S не учитывались, так как почти все они были учтены среди последовательностей 16S из-за того, что преобладающий локус охватывает оба гена.

Furthermore, a similar pattern is observed on the genus level: in all the markers examined in the current study (COI, 12S, 16S, and 18S), *Daphnia* is the most studied taxon (Figs 1, 2). In terms of total number of sequences, *Daphnia* is represented more than 2 times higher than its closest "rival", *Diaphanosoma*. Also, it should be noted that even in this top 10 (Fig. 1) more than 60 percent of the sequences belong to the daphnid genera.

In reality, cladoceran macrotaxa other than *Daphnia* are relatively poorly represented in international databases, especially groups rich in cryptic species. Even efforts of the COI sequence deposition to the GenBank and BOLD are significantly de-intensified recently as compared to the earlier enthusiastic times of the Barcode of Life initiative [Hebert *et al.*, 2003a,b; Elias-Gutierrez *et al.*, 2008; Elias-Gutierrez, Valdez-Moreno, 2008]. Accumulation of new cladoceran sequences is mainly performed by few research groups (although the necessity of such studies is obvious for many biologists dealing with this model group) and a limited number of the researchers can contribute to the cladoceran studies in particular regions [Makino *et al.*, 2017; Garlasche *et al.*, 2023; Nowosad *et al.*, 2024]. A representative set of new cladoceran COI sequences was deposited to the GenBank during last ten years by our group, or with a contribution by our group [Bekker *et al.*, 2016; Kotov *et al.*, 2016, 2021], although we do not regard barcoding as our priority. Interest in cladoceran diversity and phylogeography is shifting from western to developing countries, with a significant participation of the Chinese biologists [Deng *et al.*, 2022; Pei *et al.*, 2023] and others). Moreover, China is now among the countries with the greatest progress in the metabarcoding and eDNA studies [Zhao *et al.*, 2021].

No molecular data on the cladocerans are available for most territories of the planet, only few "spots" are studied in detail. In fact, the number of papers concerning barcoding, as well as metabarcoding, in freshwater environments grew until ca. 2021 [Elias-Gutierrez *et al.*, 2021] but stopped to grow according to a more recent graph presented by Elias-Gutierrez & Valdez-Moreno [2023]. As a result, some authors claimed that "a substantial portion of taxa that were identified to genus or species by morphological identification, but not identified using DNA metabarcoding, had zero ("no record") or ≤ 2 ("underrepresented records") reference barcodes in the BOLD or NCBI databases (63% for COI, 80% for 16S, 74% for 18S)" even in Canada being the capital of barcoding studies [Meredith *et al.*, 2021].

We have conducted a study of publication activity in the bibliographic databases, Elsevier Scopus and Google Scholar, on the topic of our interest (Fig. 3). Our data agree with the mentioned observation: we see a spectacular growth in the publications discussing both Cladocera and barcoding since 2008 until 2021 when its decline has started. The vast majority of these publications is devoted to the "traditional" barcoding using the COI mitochondrial marker. On the other hand, since the late 2010s, a steady increase in the papers featuring metabarcoding and eDNA in the context of Cladocera is observed.

We agree with Garlasche *et al.* [2023] that organization of the metabarcoding-based monitoring must be accompanied by establishment of a reference DNA library

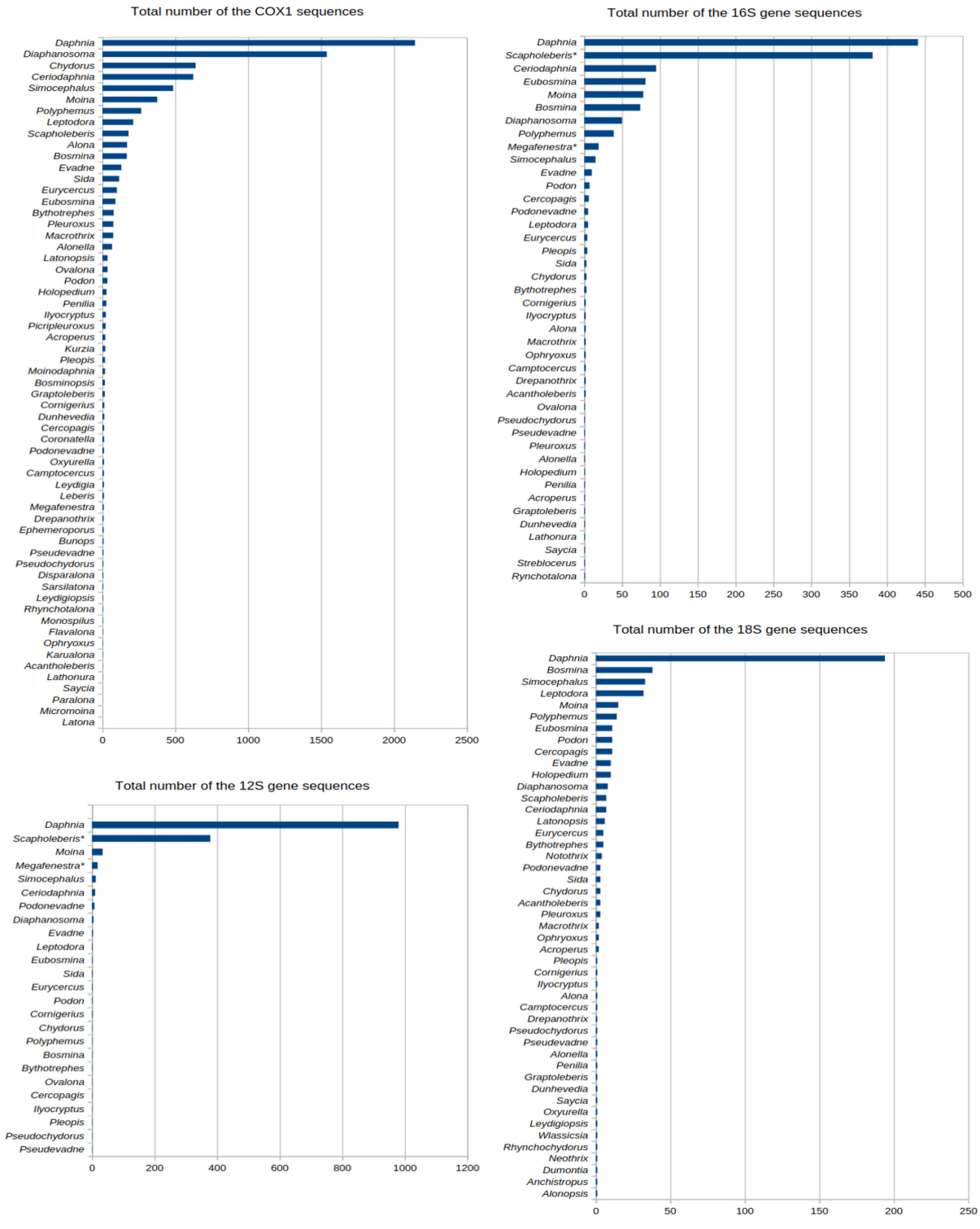

Fig. 2. Total numbers of the marker gene sequences in the Nucleotide database of GenBank by genera with at least one sequence. For each *genus* the number is obtained by searching for "*genus* [organism] AND *term*" where the term is "(COI OR COX1)" or "12S" or "16S" or "18S". For *Scapholeberis* and *Megafenestra,* the predominant locus in the database is spanned across the 12S and 16S genes, and counted separately for each one.

Рис. 2. Общее число последовательностей маркерных генов в нуклеотидной базе данных GenBank по родам, содержащим хотя бы одну последовательность. Для каждого рода число получено путем поиска по запросу "*genus* [organism] AND *term*", где *term* — "(COI OR COX1)", "12S", "16S" или "18S". Для родов *Scapholeberis* и *Megafenestra* преобладающий локус в базе данных охватывает 12S и 16S гены и учитывается отдельно для каждого из них.

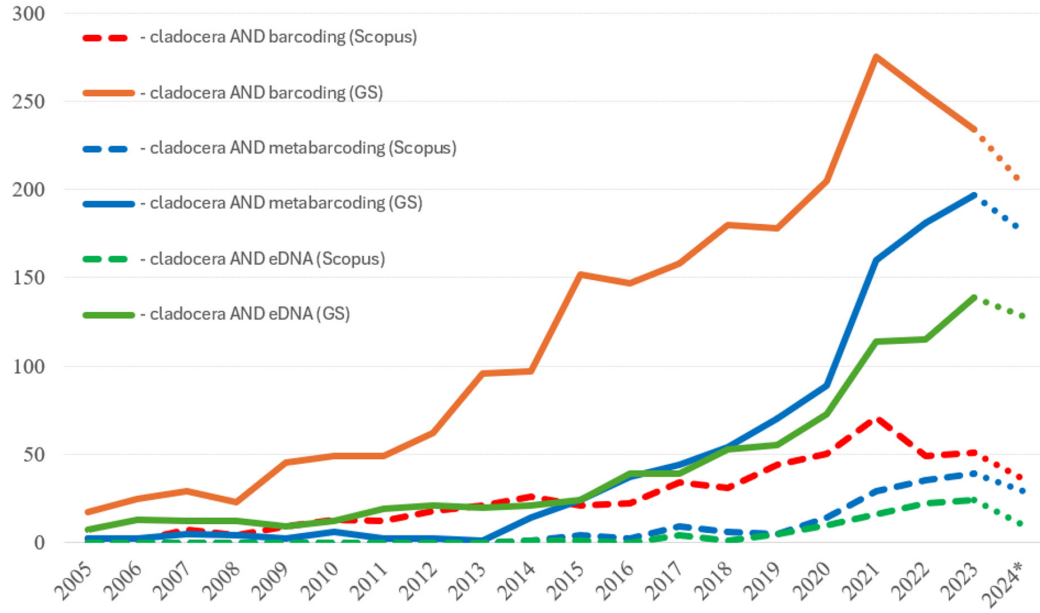

Fig. 3. Number of publications featuring different terms according to bibliographic databases Google Scholar and Scopus Elsevier on the subject. The search is performed in the titles of publications and keywords in the metadata. The data for 2024 are incomplete; they are represented for the first half of the year.

Рис. 3. Число публикаций, представленных в библиографических баз данных Google Scholar и Scopus Elsevier по данной тематике. Поиск ведется по названиям публикаций и ключевым словам в метаданных. Данные за 2024 год неполные, они представлены только за первое полугодие.

featuring local samples. Hence, most species with barcode sequences in the indigenous database could be identified by metabarcoding approach [Yang *et al.*, 2017].

Although main cladocerans known as invaders are represented in the NCBI GenBank, many other taxa are underrepresented, or fully absent in the international databases (Table 2), first of all, representatives of the family Chydoridae being the most diverse group of the Cladocera and containing c.a. 40% of all known species [Forro *et al.*, 2008]. Therefore, we barely have a chance to detect their invasions, especially cryptic ones. At the same time, our previous trans-Eurasian, trans-Holarctic, or global phylogeographic study revealed non-indigenous haplotypes, large phylogroups, and even species among the cladocerans for which such invasions were previously unknown, and no signs of such cryptic invasions were recorded by the morphologists [Garibian *et al.*, 2020, 2021; Karabanov *et al.*, 2020].

As a result, many contemporary metabarcoding studies of the plankton (including very recent and promising eRNA studies, detecting mainly genetic material from alive rather than dead specimens) use the family or generic level of the identification, with only few particular cladocerans identified up to species level [Ankley *et al.*, 2021, 2022]. Note that, in some cases, such an approach has already been able to detect non-indigenous species in the region, like two copepods found by [Ankley *et al.*, 2021]. However, others have a great chance to be missed due to their poor representation in the international databases.

Insufficient sequence database quality and nuclear mitochondrial pseudogenes. Another important hindrance to the application of the "barcoding datasets" for the metabarcoding is a set of issues concerning previously deposited sequences. First of all, there are apparent species misidentifications and misuses of the species names for populations belonging to distant locations (like other continents) to the taxon type locality making necessary to conduct a "decoding of barcoding" results in each particular case [Garibian *et al.*, 2020]. Moreover, low quality of some sequences causes big noise and could lead to misidentification, and we have no chance to check and correct them since the chromatograms are absent. Partially, this situation can be improved by special program tools for COI data cleaning and error evaluation [Nugent *et al.*, 2020]. However, it means that an investigator is supposed to check the initial dataset in the GenBank or BOLD by this tool prior to using the deposited sequences as vouchers for identification of their species. Apparently, a global check of the GenBank by its editors is urgently necessary now.

Nuclear mitochondrial pseudogenes (NUMTs) (see Leite [2012] for description of this phenomenon in insects) "appear to pose the greatest interpretational risk when short (<313 bp) amplicons are used, such as in environmental DNA studies" [Schultz, Hebert, 2022]. The statement that "inflation in OTU counts and in barcode variation were just 9 and 10%, respectively, suggesting NUMTs will not seriously distort biodiversity assessments" [Schultz, Hebert, 2022: 2897] is, in our opinion, controversial. We need to agree with Leite [2012: 301] that "NUMTs pose a major problem for taxonomic and phylogenetic studies based exclusively on barcode sequences". Lopez *et al.* [2022] also spe-

Table 2. List of the cladoceran genera present in the Holarctic without any single sequence in the NCBI GenBank.
Таблица 2. Список родов Cladocera, обитающих в Голарктике, для которых нет ни одной последовательности в базе данных NCBI GenBank.

Taxon	Taxon
Order Ctenopoda Sars, 1865	**Family Chydoridae Dybowski et Grochowski, 1894**
Family Sididae Baird, 1850	**Subfamily Aloninae Dybowski et Grochowski, 1894**
Limnosida Sars, 1862	*Anthalona* Van Damme, Sinev et Dumont, 2011
Pseudosida Herrick, 1884	*Brancelia* Van Damme et Sinev, 2011
	Korealona Jeong *et al*., 2017
Order Anomopoda Sars, 1865	*Kozhowia* Vasiljeva et Smirnov, 1969
Family Ophryoxidae Smirnov, 1976	*Nedorhynchotalona* Kotov et Sinev, 2011
Parophryoxus Doolitle, 1909	*Nicsmirnovius* Chiambeng et Dumont, 1999
Family Chydoridae Dybowski et Grochowski, 1894	*Parakozhowia* Kotov, 2000
Subfamily Chydorinae Dybowski et Grochowski, 1894	*Phreatalona* Van Damme, Brancelj et Dumont, 2009
Estatheroporus Alonso, 1990	*Tretocephala* Frey, 1965

cially claimed that the nuclear-encoded mitochondrial pseudogene contamination is a very serious problem for genomic methods (gDNA) as "mock community analyses showed that the use of gDNA mitochondrial cytochrome *c* oxidase I (mtCO1) amplicons inflates species richness". They applied a metatranscriptomic approach and found that "significantly more amplicon sequence variants, nucleotide diversity, and indels were observed with gDNA amplicons than with cDNA, indicating the presence of putative NUMT pseudogenes". Such pseudogenes have been already found in the cladocerans [Kowal *et al.*, 2020] but there is no information on pseudogenes in the non-daphniid genera. Again, special software tools could be proposed for the NUMTs search and removal [Flamingh *et al.*, 2023], but nobody uses them in the cladoceran studies, including metabarcoding.

Finally, we have to underline weak representation of the sequences from invasive and potentially invasive cladoceran taxa in the Genbank and BOLD. Among the species expanded their distribution ranges listed by Kotov *et al.* [2022], eight taxa lack any COI sequences:

Limnosida frontosa Sars, 1862
Simocephalus hejlongjiangensis Shi et Shi, 1994
Wlassiscia pannonica Daday, 1904
Biapertura ossiani herricki (Sinev, 2013)
Disparalona striatoides (Šrámek-Hušek, 1946)
Flavalona rustica (Scott, 1895)
Ovalona weinecki (Studer, 1878)
Phreatalona protzi Hartwig, 1900.

Alongside the previous issue, wrongly deposited "vouchers" could be a critical obstacle for the earlier invasion detection.

Problems with locus selection

mtDNA marker COI. A very (if not the most) important problem is the locus selection for the metabarcoding. The most complete available data (e.g. see Figure 3 for the genera representation of different markers in the GenBank) concerns the mitochondrial COI gene used in traditional "DNA barcoding" sensu Hebert *et al*. [2003a,b], more specifically, the Folmer region (ca. 700 bp in the beginning of the gene). Since the usual length of a read in a short-read sequencing does not exceed 250 bp and paired reads are used, up to approximately 0.5 kb subregion has to be chosen. At the same time, a significant variability does not allow providing a universality to so-called "universal" primers. Several authors proposed other primers for COI metabarcoding [Leray *et al.*, 2013; Schroeder *et al.*, 2021].

In any case, due to the absence of good reference libraries on non-daphniids, researchers use identification up to the genus level, moreover, even such identifications as "*Macrothrix*" of "*Ilyocryptus*" [Yang, Zhang, 2020; Cen *et al.*, 2023] could be referred to other macrothricid-like genera lacking vouchers in the GenBank. A very typical situation is observed in recent papers on the cladoceran metabarcoding: only few taxa from the total sample or eDNA are identified based on COI at the species level, while others — at the genus level [Yang *et al.*, 2017; Yang, Zhang, 2020; Valdez-Moreno *et al.*, 2021; Liang *et al.*, 2024]. In some cases, only identification at the family level is used [Clarke *et al.*, 2017; Martin *et al.*, 2021; Qiu, Liu *et al.*, 2022], and such resolution level is regarded as sufficient for metacommunity studies.

Apparently, studies based on COI are most effective in case of a well-studied territory, especially, Europe [Vogelmann *et al.*, 2024], where the plankton could be identified up to species level in the total samples, water and sediments. Kiemel *et al.* [2023] made such a study for plankton, but note that these authors said nothing about littoral cladocerans (i.e., in sediments) — we do not know, did the authors simply ignore them, or they were really absent?

In the case of the neritic zone of the World Ocean with only a few cladoceran species and which are represented in the GenBank, their identification up to species level works well [Stefanni *et al.*, 2018; Zamora-Terol *et al.*, 2020; Garcia-Vazquez *et al.*, 2021; Singh *et al.*, 2021; Song, Liang, 2023]. The same can be said about well-known invasive species as targets of metabarcoding

studies. For example, Mychek-Londer *et al.* [2020] successfully detected DNA of *Bythotrephes longimanus* and *Cercopagis pengoi,* harmful invasive species in the Great Lakes basin, in fish stomach contents. Moreover, it was possible to detect presence of *B. longimanus* in sedDNA using COI primers, which is crucial for understanding of the invasion history of this taxon and similar approach could be applied to other taxa in the future. On the other hand, it is not guaranteed that short COI fragments could identify some cases of cryptic invasions, especially, in the case of invaders being closely related to indigenous species, or invasions of intraspecific lineages [Karpowicz *et al.*, 2024].

Sometimes the COI primers do not work for cladocerans, although other primers detect a significant generic and species number. Zhao *et al.* [2021] detected during different seasons 29 OTUs belonging to the Cladocera using 18S primers, and no one cladoceran OUT during any seasons using COI. We had a similar situation in our studies (our data, in prep.). It could be explained by the fact that such primers usually developed for a wide range of zooplankton taxa and oftentimes biased towards marine diversity, where cladoceran representation is minuscule. As mentioned, significant variability of the COI gene is an insurmountable obstacle to the design of the universal primers.

mtDNA markers 12S and 16S. These prospective, but rarely usable loci of mitochondrial DNA, although less variable than COI, provide better resolution than 18S. Gao *et al.* [2020: 62] found that "16S primer had better specificity to zooplankton species, where 88.1% of 16S OTU came from zooplankton", and they were able to identify some cladocerans up to species level in their total zooplankton samples. At the same time, in recent studies, identification is performing mainly up to genus level [Novotny *et al.*, 2021; Novotny, 2021]. The main drawbacks are insufficient representation in the databases (Fig. 3), and inability to discern some very closely related species and lineages due to the limited variability as compared to COI.

nDNA marker 18S. This locus is very "friendly" in terms of the successful analysis using "universal" primers. However, it demonstrates a low variability (and, therefore, a low resolution), and a mediocre representation in the international databases. Sometimes it was used for detection of major planktonic macrotaxa, like cladocerans in total [Banerji *et al.*, 2018], or their families [Clarke *et al.*, 2017; Di Capua *et al.*, 2021; Novotny, 2021; Qiu, Lu *et al.*, 2022]. Using this marker, it is difficult to analyse complex and taxonomically diverse communities because, e.g., "a single sequence divergence threshold does not always generate good correspondence between OTU number and species richness" [Brown *et al.*, 2015]. Ji *et al.* [2022: 1] concluded that "only ~56% of the zooplankton genera reported in Korea could be detected based on the 18S rRNA gene", moreover, the results of metabarcoding and microscope identification (even at generic level!) were sometimes different. Therefore, the resolution of such studies is unacceptably low. Furthermore, Monchamp *et al.* [2022] found "a significant correlation between the relative abundance of zooplankton families identified based on SSU rRNA gene prediction and morphology", but "differences in congruence between metagenomes and morphological identifications were detected when varied bioinformatic approaches were applied to the presence-absence data". In our opinion, the 18S data could be only used as supplementary to the metabarcoding with the mitochondrial markers.

Nevertheless, we need to admit that sometimes application of the 18S only was very successful [Zhao *et al.*, 2021]. Also, this marker has been already used for detection of the invasive species from different animal taxa, including cladocerans *Cercopagis pengoi* and *Daphnia galeata*, based on eDNA [Brown *et al.*, 2016]. Therefore, this locus could be sensitive enough to detect some non-indigenous taxa, in spite of its low resolution.

Multiple markers. In many studies, a multiple marker approach (either combining results of independent runs, or using multiplex PCR) is tested, like a combination of 18S+16S [Stefanni *et al.*, 2018; Zamora-Terol *et al.*, 2020], 18S+COI [Stefanni *et al.*, 2018; Kiemel *et al.*, 2023], 18S+12S+COI [Schallenberg *et al.*, 2023], or 18S+16S+two fragments of COI [Meredith *et al.*, 2021]. Zhang *et al*. [2018] strongly recommended marker multiplexing (i.e. using combination of different markers in a single PCR) for metabarcoding of the zooplankton communities. They found that "the species detection level was significantly improved to 89–93% when both markers were used". As far as we are concerned, since multiple markers can cover blindspots of each other, we believe they should be preferred in biodiversity research and ecological monitoring whenever it is feasible.

Conclusions

We are sure that after some years the eDNA methods will form a basis for the monitoring of indigenous and non-indigenous aquatic taxa, and such methods will be officially recommended by the environmental authorities of different countries. However, now we need to resolve the main problems concerning their use. If the problems of the low quality of the database entries, like misidentifications of taxa or presence of pseudogenes disguised as proper vouchers, etc. will not be resolved, many researchers will be misguided, and many automated pipelines will give noisy or outright wrong results. Although, we have to admit it can be done only by the editors of the databases, or, alternatively, new reference databases could be created by the community.

On the other hand, we believe that current efforts of the cladocerologists need to be focused on the filling of the GenBank with sequences of all the loci widely used in the metabarcoding from all the known cladoceran genera and species in different regions of the world. Such efforts could be coordinated with the complete mitogenome (and full genome) sequencing initiatives which allows forming the basis for future eDNA studies, including, but not limited to, the metabarcoding.

Now is the best time to start this job, but needless to say that efforts of several scientific groups are necessary.

International cooperation and a responsive community are indispensable in such an endeavour.

Compliance with ethical standards

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

Ethical approval: No ethical issues were raised during our research.

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