DNA Barcoding, Environmental DNA and an Ongoing Attempt of Detecting Biodiversity in Lake Kasumigaura

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Abstract

Conservation of freshwater ecosystem is in urgent need because it provides abundant ecological services to humans. Data of biodiversity obtained from a long-term monitoring are essential for planning conservation activities. However, since species identification needs expertise in taxonomy, collecting biodiversity data under a standardized monitoring protocol requires a great effort to overcome taxonomic difficulty. Recently developed methodologies using DNA barcoding and environmental DNA (eDNA) are expected to overcome the difficulty in a long-term monitoring. DNA barcode is a nucleotide sequence of a genome that has good resolution in species identification. DNA barcoding allows us to identify species even without taxonomical expertise. DNA extracted from environmental samples such as soil and water is called eDNA, that contains DNA of a variety of organisms from microbes to macroorganisms. Analysis of eDNA yields two kinds of data: (i) occurrence and abundance data of specific organisms obtained by species-specific primer and quantitative PCR, (ii) a list of a wide variety of species inhabiting an environment, obtained by species-universal primer, next generation sequencer and a reference database of DNA barcodes. By applying those methodologies to biological monitoring of freshwater, data could be kept in high quality for a long period. Moreover, records of biodiversity can be retrospectively analyzed from eDNA, because eDNA can be semi-permanently stored. Now we are installing the eDNA methodology on the long-term monitoring of Lake Kasumigaura. For detecting biodiversity of animals in the lake, a preliminary sequencing of cytochrome *c* oxidase subunit I (*COI*) of eDNA in the lake water was conducted. As a result, species of zooplankton were detected, but benthos and fish were hardly detected. This suggested that sequences of benthos and fish should be analyzed by other techniques such as the use of specific primers, which is different from the analytical methodology of zooplankton.

Introduction

Although knowledge of ecosystem dynamics has been accumulated in recent years, predicting a changing ecosystem and proposing conservation plans for it are still challenging. To increase reliability of ecosystem estimation and validation for the conservation activities, a long-term monitoring of ecosystem is needed. However, keeping collecting meaningful data, especially those of biodiversity, at multiple sites and/or at certain frequency, takes a great deal of effort; for it requires expertise in the identification of diverse organisms. In terms of convenience and objectivity, a recently developed methodology using DNA sequences offers a solution for biological monitoring, especially for water monitoring. In this paper, we first mention the issue of biodiversity monitoring in freshwater ecosystem. Second, we describe the applicability of environmental DNA (eDNA) for monitoring of freshwater biodiversity and the importance of DNA barcoding that is essential for analyses of eDNA. Finally, some preliminary results from our ongoing eDNA survey in Lake Kasumigaura presented.

Biodiversity of freshwater

Although a number of ecosystems on earth are facing an urgent necessity of conservation of biodiversity, conservation of freshwater ecosystem is behind that of terrestrial and marine ecosystems. The monitoring data of the increase and decrease in vertebrates populations from 1970 to 2010 (Living Planet Index = LPI) showed that decreased populations were 76% in freshwater species, and 39% in both terrestrial and marine species (WWF 2014). Freshwater ecosystem accounts for small areas on earth; 0.8% of the earth surface and 0.01% of water. However, it is biologically diverse; 6% (100,000 species) of described species, one third of vertebrate species, and 40% (13,400 species) of fish species inhabit freshwater (Dudgeon *et al.* 2006). Biodiversity of small organisms such as plankton and benthos in freshwater is expected to be quite high, but it is considered to be underestimated partly because of their difficulty in morphological identification.

In biodiversity monitoring, morphological identification of organisms for data classification generally needs expertise even within small taxa. This identification methodology highly relies on an observer's skill, and even for an expert, identification of smaller organisms requires considerable time and energy. Moreover, we should carefully handle the data obtained from morphological identification because it sometimes lacks objectivity due to conflicting and different interpretation of taxonomical keys. These disadvantages set limits on the quality and objectivity of monitoring data for biodiversity to be standardized for a long period or multiple sampling sites in a broad range of area at the same time. In addition, some reports pointed out that a recent decrease in the number of taxonomists might cause the difficulty in securing experts in taxonomy and training new taxonomists (Hopkins and Freckleton 2002, Joppa *et al.* 2011).

Recent analyses of eDNA from water using DNA barcodes are expected to compensate for the disadvantages of morphological identification. Using eDNA from water would decrease the difficulty of sampling and species identification and increase the objectivity of its data. In the following section, we will describe the present situation of DNA barcoding and the recent application of eDNA for detecting biodiversity.

DNA barcoding

In this decade, DNA barcoding has been proposed for the objective identification of organisms (Hebert *et al.* 2003a). DNA barcode is a sequence in a certain region of a genome and it has sufficient nucleotide differences to distinguish species. DNA barcodes generally used are *16S rRNA* for bacteria (Caporaso *et al.* 2012), ribosomal RNAs and internal transcribed spacer (ITS) for eukaryotes (Pawlowski *et al.* 2012, de Vargas *et al.* 2015), cytochrome oxidase c subunit I for animals (*COI*, Hebert *et al.* 2003b) and *rbcl* or *matK* for plants (CBOL Plant Working Group *et al.* 2009). International Nucleotide Sequence Database Collaboration (INSDC) is the biggest and the most popular database for referring DNA barcode, and Barcode of life database (BOLD) systems (Ratnasingham and Hebert 2007) and Silva for ribosomal RNA (Quast *et al.* 2013) would provide more reliable data than INSDC.

The significance of identification using DNA barcode lies in its objectivity, which relies on the information of nucleotide sequences. Whether or not one is a taxonomist, anybody who has a technique of DNA experiment is able to obtain exactly the same nucleotide sequence from the target DNA sample. Moreover, DNA sequence itself can be a powerful tool for distinguishing cryptic species that are not morphologically discriminated. Since testing machines and convenient commercial reagent kits have been developed for more general use, techniques using DNA have no longer been reserved only for some researchers. Therefore, to receive the benefit from the versatility of DNA barcoding, we should carefully handle sequence data for the next three reasons.

First, sequence data deposited in a database are not absolutely true. Users of DNA barcode are exposed to the risk of possibly invalid interpretation of taxonomy. We should carefully analyze the data by checking ecological information, confirming clusters such as barcode index number (BIN) on BOLD system (Ratnasingham and Hebert 2013), and phylogenetic analyses, especially when researchers do not have sufficient expertise in the treated group or taxa of organisms.

Second, those databases often provide only names of family, order or classes but not species, because accumulating DNA barcodes is still under way in the world. It has been noticed that promoting accumulation of DNA barcoding is an urgent issue (Jinbo *et*

al. 2011). However, research for DNA barcoding is still progressing slowly in many regions of the world and for various taxa, because great effort is needed for sampling and their morphological identification (Jinbo *et al.* 2011). Unfortunately, it can be said that accumulated DNA barcodes for Japanese species are relatively scarce despite a number of research on molecular phylogeny and biogeography. According to Union of Japanese Societies for Systematic Biology (2003), the number of described Japanese biota species was 89,088 (excluding bacteria). In BOLD systems in March 2016, DNA barcodes of 6,864 species and 4,574 BINs in Japan were shown and they are only 7.7% and 5.1% of the total number of described species. In addition, the number of DNA barcodes in Japan is also relatively small when compared to other countries or taxonomic groups where DNA barcoding project has been energetically proceeding (Fig. 1). Considering the richness in endemic species and subspecies in the insular country, lack of DNA barcoding data must limit the applicability of recent developed methodologies such as metabarcoding using environmental DNA (eDNA). Therefore, for identifying more species, it is necessary to actively promote construction of DNA barcode database.

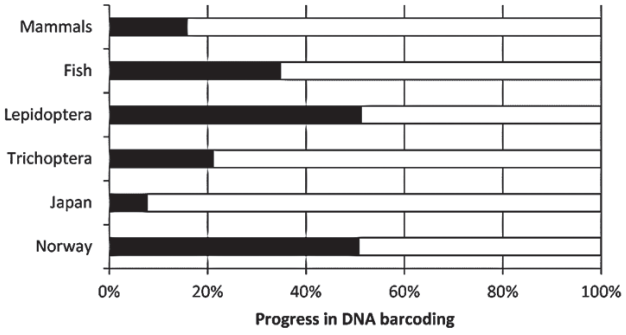

Fig. 1 Progress in DNA barcoding in the projects of taxa and countries. Black indicates frequency of species that was already DNA-barcoded. White indicates species that was described or targeted for DNA barcoding but have not barcoded yet. Data were obtained from the following sites on March 25, 2016. For mammals, iBOL mammalia barcode of life (http://www.mammaliabol.org/); for fish, Fish barcode of life (FISH-BOL, http://www.fishbol.org/); for epidoptera, Lepidoptera barcode of life (http://lepbarcoding.org/index.php); Japan, BOLD systems; Norway, Norwegian barcode of life (NorBOL, http://www.norbol.org/).

Absence of theoretical threshold for nucleotide differences distinguishing taxa often makes interpretation of taxonomy difficult. Genetic distance between species is empirically shown to be under 10 %, which in general depends on their taxa and gene regions (Hebert *et al.* 2004, Moritz and Cicero 2004, Jinbo *et al.* 2011). Therefore, if the genetic distance between sequences of two species differs by more than 10%, those species can be distinguished from each other. Conversely, when an unknown sequence of a species is perfectly matched to that of an identified species, both species can be considered as identical. However, when the difference of genetic distance is apart from 0 or 10 %, it often requires great effort to determine whether or not those species are matched. Hence, the intermediate difference of genetic distance between sequences should be carefully treated on the basis of specialized knowledge on taxonomy and molecular phylogeny.

Besides the above points, researches on biodiversity using DNA barcoding, especially metabarcoding using NGS, provide us extreme amount of information with amazingly high efficiency (de Vargas *et al.* 2015, Leray and Knowlton 2015). On the other hand, we emphasize the continuous efforts of taxonomists even though DNA barcoding research proceeds. Expertise of taxonomist is absolutely essential for accumulation of reliable information of DNA barcoding. Although most DNA barcodes used at present include taxonomical information, they do not have ecological or plastic traits such as color, shape and behavior, which are generally found by taxonomists.

Environmental DNA

Environmental DNA (eDNA) is an inclusive term for DNA extracted from environmental samples such

as water and soils (Bohmann *et al.* 2014, Barnes and Turner 2015, Thomsen and Willerslev 2015). Environmental samples are supposed to include whole body of microorganisms such as bacteria and planktons and also pieces of tissues, secretion and stools of larger organisms inhabiting the environment (Barnes and Turner 2015). The eDNA extracted from only a small amount of an environmental sample is filled with sequence information of wide range of organisms than had been expected before (Ficetola *et al.* 2008, Thomsen *et al.* 2012, Thomsen and Willerslev 2015). Recent analyses of eDNA related to biodiversity fall into two major groups. The one analytical methodology is the detection of one to several specific species by using species-specific primers and quantitative PCR (qPCR). This methodology has often been applied to detect the distribution of endemic species (Fukumoto *et al.* 2015) or invasive species (Takahara *et al.* 2013, Uchii *et al.* 2015). Since a bottle of water is enough for extracting eDNA, less damage will be caused in the field survey and less effort can be needed for the field work. Moreover, the data obtained by qPCR using specific primers for a target species can show not only its presence of species qualitatively but also its biomass quantitatively, as Yamamoto *et al.* (2016) reported that the abundance of Japanese jack mackerel could be estimated even in a bay. The other analytical methodology, called metabarcoding, sets the target at a broad range of organisms and exhaustively detect biodiversity in an environment by obtaining massive sequences using universal primers for different species and next-generation sequencer (NGS) (de Vargas *et al.* 2015, Miya *et al.* 2015). This methodology is less quantitative than qPCR, because its efficiency in PCR amplification with universal primers differs among species. However, this universality of primer annealing also enables us to detect unexpected species and the universality of metabarcoding with eDNA provides massive information of biodiversity including rare and unrecognized species. In both methodologies, integrity of the database of DNA barcodes is essential to design specific or universal primers and refer unknown sequences.

One of the advantages of this biological monitoring methodology using eDNA is the compact space for the storage of eDNA samples in micro tubes or computerized data of sequences. Moreover, DNA samples and sequence data can be stored semi-permanently in laboratories. In the traditional field studies, preservation of organismal samples often needs harmful substance and space for storage and therefore samples or data of non-target or unrecognized organisms at the treatment are generally neglected and not stored for a long time. In contrast, an eDNA sample and its sequence data from metabarcoding involve information of non-target or unrecognized organisms at the time of sampling and we are able to reanalyze them retrospectively. Simple sampling process is also the advantage of monitoring by eDNA, because sampling only a small volume of water needs no special technique. This easy sampling process enables to increase the number of monitoring sites and frequency of monitoring. Some recent reviews of eDNA illustrated the detection and analysis of eDNA and some reviews and articles also discussed unsettled problems and arguments on the reliability of eDNA (Bohmann *et al.* 2014, Rees *et al.* 2014, Barnes and Turner 2015, Rees *et al.* 2015, Roussel *et al.* 2015, Thomsen and Willerslev 2015).

Monitoring Lake Kasumigaura

Lake Kasumigaura is the second largest lake in Japan and located in the Kanto Plain where Tokyo and other large cities gather together. The lake provides us important ecological services such as water resources for drinking, agriculture and industries, and fishery, and leisure and purification of water. This close relationship of water with humans strongly affects the lake, and serious problems such as declining quality of water, blooms of blue-green algae, and invasion by alien species have been raised for many years. To understand the mechanism of ecosystem in Lake Kasumigaura, a research group of National Institute for Environmental Studies has monitored water quality and biodiversity since 1970's. Qualitative and quantitative data of bacteria, phytoplankton, zooplankton, benthos and fish are publically released on the website (http://db.cger.nies.go.jp/gem/moni-e/inter/GEMS/database/kasumi/index.html). Since it is difficult to keep standardized level of the taxonomic resolution for such a broad range of organisms for a long period, we have started to apply eDNA for the monitoring. In the rest of this paper, we will show preliminary data for animals obtained from metabarcoding of eDNA in Lake Kasumigaura and provide our perspectives on the outcome from the data analysis.

Figure 2 shows an example of metabarcoding of eDNA extracted from 250 ml water of Lake Kasumigaura on Oct. 6, 2014. The PCR reactions amplified with universal primers for *COI* gene target for animals were analyzed using NGS (IonPGM, Life Technologies). Although universal primers for animals were used, bacteria and phytoplankton were also identified. For animals, zooplanktons of Branchiopoda, Maxillopoda and Monogononta, and benthic insects were detected. Those detected zooplankton covered the main species in Kasumigaura (data not shown) and this result suggested the practical use of eDNA for monitoring biodiversity of zooplankton. On the other hand, more than 75% of contigs were not identified by Blast search. There must be a few chimeric sequences that were not removed at the assembling process, and lack of DNA barcodes in the database was considered to mainly cause the "unknown" contigs. Therefore, we are now promoting the DNA barcoding of organisms that live in Lake Kasumigaura, and the data of algae and chironomids are sequentially published on the database.

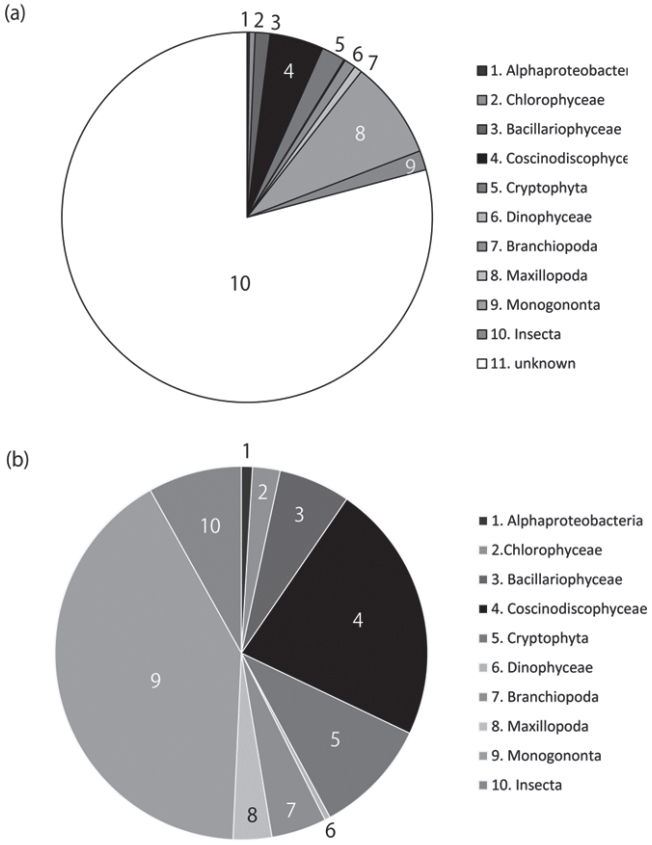

Fig. 2 Taxa detected in eDNA of water from Lake Kasumigaura. The *COI* sequences were assembled and identified by Blast search in the program package, Claident (Tanabe 2013). (a) All contigs, (b) contigs excluding unknowns.

The benthic organisms accounted for a large part of their biomass, but rarely detected from the metabarcoding of eDNA from water sample. This result might be caused by low concentration of their DNA in eDNA samples and relatively low efficiency in PCR amplification with the universal primers when compared with zooplankton. For further investigation, we will examine the water depth for sampling and design more compatible primer sets for chironomid and oligochaete species that were reported to have the largest proportion of biomass at the bottom of Lake Kasumigaura.

Fish were hardly detected from the metabarcoding of *COI* using animal universal primers (Fig. 2), which might be due to less quantity of DNA than zooplankton and inefficiency of annealing with universal primers used in this study. Although the primer set was confirmed to amplify *COI* sequences in more than

90% of fish species in Kasumigaura (data not shown), the relative efficiency would be lower than other organisms such as zooplankton. Therefore, metabarcoding of fish species from eDNA should be conducted by other systems using specific primers for fish, such as MiFISH (Miya *et al.* 2015).

Metabarcoding can be applied not only to eDNA of water body, but also to merged samples or DNA samples of individual organisms. Either the DNA solutions extracted all together from individual specimens or the mixture of DNA samples that were individually extracted from each specimen could be subject to metabarcoding of NGS (Yu *et al.* 2012). We also had preliminary data of metabarcoding for the mixture of DNA samples extracted individually from 71 species of Japanese chironomids. We mixed up the DNA samples of different concentration in equal volume into one tube and their *COI* was amplified with universal primers used above. We analyzed two replicates of PCR reactions and obtained 36 species from 47,860 reads and 29 species from 117,240 reads, respectively (Fig. 3). In total, 39 species (55%) were detected, while 32 species (45%) were not detected (Fig. 3). The correlation between the number of contigs and concentration of DNA was not significant (P=0.7983, Fig.4), and as same as the dataset excluding species without detected contigs (P=0.6931). The result might reflect the different affinity of universal primers among species rather than the concentration of each DNA sample used in the mixture. From our preliminary data, we found out that metabarcoding of DNA mixture needed improvement. Developing the primer set must be effective and exchanging the order of mixing DNA and PCR could be another way to

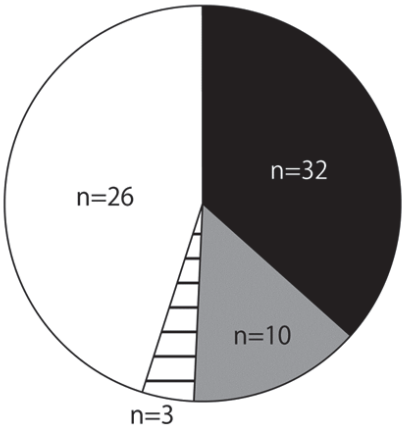

Fig. 3 Success in species identification of chironomids from the pooled DNA using NGS. DNA samples of each species were extracted individually and mixed with each volume. The result of two replicates is shown. In total of 71 species, species detected in both replicates are shown in black, those detected only in replicate 1 and 2 are shown in gray and stripes, respectively, and white shows species that were not detected from neither of replicates.

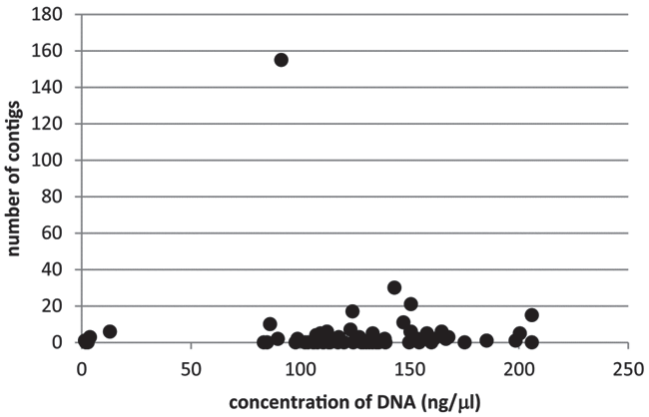

Fig. 4 Concentration of each DNA and its detected number of contigs in the NGS analysis of mixed DNA samples shown in Fig. 3.

improve the efficiency of detection.

There are several points of particular concern for the practical use of eDNA to monitor Lake Kasumigaura. First, a pore-size of filters and amount of filtering water must be critical, which were already presented in previous studies (e.g. Turner *et al.* 2014, Barnes and Turner 2015). In general, eDNA of water sample was extracted from a filter that traps eDNA and microbiota through filtration of the water sample. In most of those studies, filtering water with the pore size of 0.2 μm was recommended, but those filters were easily clogged before going through enough amount of water sample from Lake Kasumigaura. Second, the efficiency of detecting biodiversity should be considered in the application of eDNA to a long-term monitoring. Since there is no perfect primer set to detect all taxa at one time, minimal number of primer sets for target biodiversity should be investigated. For other general issues such as contamination, spatial and temporal scales should be also discussed (Thomsen and Willerslev 2015).

In recent years the efficiency and sensitivity of monitoring using eDNA have become more recognized. However, this does not necessarily mean the replacement from the traditional monitoring methodology based on morphology to the new monitoring methodologies. Applying eDNA in combination with DNA barcoding would enhance the efficiency of detecting biodiversity, which traditional monitoring has not achieved. On the other hand, the abundance and biomass of species are well estimated from the traditional sampling of organisms itself compared with those novel methodologies, at least for now. DNA barcodes can tell us the name of organisms, whereas there are important traits that are not seen by DNA barcodes. The progress in the analysis of eDNA will overcome those difficulties in the future. Until then, it is necessary to understand both advantages and disadvantages of monitoring based on tradition or eDNA and handle them properly.

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