Doctoral Thesis

Fundamental knowledge and novel applications of environmental DNA analysis for aquatic ecology

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Department of Environmental Solution Technology
Graduate School of Science and Technology, Ryukoku University

T16D004 Satsuki Tsuji

Abstract

Environmental DNA (eDNA) analysis has been rapidly developing as a new tool for the biomonitoring of aquatic macroorganisms. Environmental DNA analysis is used for various research challenges, and its usefulness and availability are demonstrated. On the other hand, the shortage of the basic knowledge on eDNA dynamicsin eDNA analysis sometimes limits a confident interpretation of the analysis results. Therefore, this study performed with the purpose of 1) the improvement of the basic knowledge on eDNA degradation and 2) the development of a new method to expand the applicability of eDNA analysis.

In Chapter I, almost all published studies that detected aquatic macroorganisms using eDNA analysis were reviewed to organize information on the methods used in each analysis step of eDNA analysis. This chapter contributed to the understanding of the current situation of eDNA analysis by organizing each method based on the frequency of use and summarizing the characteristics and future challenges of major methods.

In Chapter II, the eDNA degradation rate was examined in relation to the influence of water temperature. The purpose of this chapter was accumulation of the knowledge about eDNA dynamics aimed at optimizing of analytical method of eDNA. First, river water was sampled and eDNA concentrations were determined for ayu (*Plecoglossus altivelis altivelis*) and common carp (*Cyprinus carpio*) at seven time points, over a 48-h period, and at three different water temperatures. The degradation of eDNA was modeled for each species using an existing exponential decay model with an extension to include water temperature effects. Additionally, the relationship between eDNA concentration and bacterial abundance were examined by spiking purified genomic DNA of the common carp into aquarium without the target species. As a result, Environmental DNA degradation was accelerated at higher water temperatures, while bacterial abundance did not have a significant effect on eDNA degradation. These results suggest that the proper treatment of this temperature effect in data interpretations and adjustments would increase the reliability of eDNA analysis in future studies.

In Chapter III, to develop the method for simultaneously detecting several species, the real-time multiplex PCR method was applied for eDNA analysis. Currently, metabarcoding and species-specific PCR are being used for eDNA analysis to detect target species. However, metabarcoding is not cost-effective when only a few species are targeted because of the wasted consumption of read due to amplification of non-target species DNA. On the other hand, species-specific PCR requires tests to be repeated multiple times resulting in consuming more DNA templates, and experimental consumables. Here, the species-specific primer-probe sets for two species of Japanese medaka (*Oryzias latipes* and *O. sakaizumii*) was developed, and it used in the real-time multiplex PCR for simultaneously detecting a few species. In aquarium experiment, even when the species abundances were biased, both species were simultaneously detected in all samples. In a field survey, eDNA analysis and capture survey produced consistent results in all sampling sites, including sites with low fish densities. eDNA analysis using real-time multiplex PCR can be easily applied to other aquatic organisms and genes, enabling a more cost-effective distribution survey of multiple target organisms. Therefore, it is expected that the eDNA analysis with real-time multiplex PCR will be a useful tool for study such as the detection of several species which have a symbiotic relationship and the monitoring of spawning activity based on the change of the abundance ratio of mitochondrial DNA and nuclear DNA.

In chapters IV and V, new analytical technique was proposed to estimate genetic diversity by sequencing eDNA contained in water samples using high throughput sequencing (HTS). Estimation of the intraspecific diversity using conventional methods is generally laborious and invasive because target organisms need to be captured for tissue sampling. Therefore, it is expected that the development of more efficient and non-invasive method promotes estimating the intraspecific diversity in ecosystem management.

In Chapter IV, it was suggested that an analytical technique combining the denoising using amplicon sequence variant (ASV) method and the selection of haplotypes based on detection rates among multiple library replications is useful for eliminating false positive haplotypes. A mixture of

rearing water including nine haplotypes of ayu mitochondrial D-loop region was used as an eDNA sample, and the 15 replicates of sequencing libraries were prepared. All library replications were sequenced by HTS, and the total number of detected true haplotypes and false positive haplotypes were compared with and without the denoising using the ASV method. As a result, the use of the ASV method considerably reduced the number of false positive haplotypes from 5,692 to 31, and it detected 8/9 true haplotypes. In addition, eight true haplotypes were detected in all 15 library replicates; however, false positive haplotypes had various detection rates from 1/15 to 15/15. Thus, by removing haplotypes with lower detection rates than 15/15, the number of false positive haplotypes were more reduced from 31 to seven. The analytical technique proposed in this study successfully eliminated most of false positive haplotypes in the HTS data obtained from eDNA samples, which allowed us to improve the detection accuracy for evaluating intraspecific diversity using eDNA analysis.

In Chapter V, the detection power of the proposed analytical technique in Chapter IV for estimating intraspecific diversity was evaluated by examining whether haplotypes of ayu detected by a conventional method can be detected from field water samples. A water sample and ayu specimens were collected at a river on the same day. Water sample was divided into 20 filters each of which had five first PCR and second PCR replications to examine possible miss sampling and amplification of scarce haplotypes. Out of the 42 haplotypes obtained from 96 specimens by a conventional method, 37 haplotypes were detected from the 20 filters. The detection rate of each haplotype among filters or library replications increased with the number of specimens that owned corresponding haplotype. Importantly, after eDNA analysis of three filters which had five library replications by eDNA analysis, the increase the number of detected haplotypes that correspond with those of 96 specimens became gentle; thus, eDNA analysis was considered to be enough sensitive to decrease replication numbers. The detection power of eDNA analysis for estimating intraspecific diversity of wildlife populations was shown to have great potential as a practical tool for researchers and natural resource managers.

The final conclusion of this research was summarized in Chapter VI. As a whole, this study focused on the analysis method of eDNA analysis for detecting aquatic macroorganisms. The trend of analysis methods used in each analysis step was revealed by reviewing most of papers. In addition, this study showed that the eDNA degradation rate is greatly influenced by temperature. Furthermore, two new analytical techniques for simultaneous detecting of several species and estimating of intraspecific diversity were proposed to expand the availability of eDNA analysis. These studies will contribute to the optimization of eDNA analysis and expand the availability of eDNA analysis.

要旨

近年、大型水棲生物を環境中から検出するための新しい手法として環境 DNA 分析が急速に発展している。環境DNA分析は多様な研究課題に利用され、その有用性が示されている。しかし一方で、環境 DNA に関わる基礎知識の蓄積が追いついておらず、利用者の間でその利用方法についての混乱が起きたり、結果の信頼性についての疑念がぬぐい切れないケースがあるなどの懸念が生じている。そこで本研究では、1）eDNA 分析の結果に影響を与えるeDNA 分解に関する基礎知識を拡充し、2）eDNA 分析の適用性を広げる新しい方法を開発することを目的として研究を行った。

第一章では、環境DNA分析の各分析ステップで用いられる手法についての情報を整理するために、国際誌で発表された環境 DNA 分析を用いた大型生物の検出に関するほとんど全ての論文をレビューした。各手法を使用頻度に基づいて整理し、主要な手法について特徴や今後の課題をまとめることにより、環境 DNA 分析で用いられている手法の現状を整理した。

第二章では、環境 DNA の動態に関する基礎知識を得るために、環境 DNA の経時的分解速度に対する水温の影響を検討した。まず、採取した河川水を 3 つの異なる温度でインキュベートし、48 時間後まで経時的に試料水中に含まれるアユ (*Plecoglossus altivelis altivelis*) およびコイ (*Cyprinus carpio*) の環境 DNA 濃度を測定した。また、既存の指数関数的減少モデルに水温効果を新たに組み込み、各種について時間経過と水温を考慮した環境 DNA 分解の非線形モデル式を作成した。環境DNAの分解速度が温度依存性を引き起こす原因に、水温による微生物量増加が考えられる。そこで、微生物による DNA 分解への影響を調査するために、コイが入っていない水槽の飼育水にコイ肉片由来の精製 DNA を添加し、添加直後および 3 つの異なる温度で 12,24 時間インキュベートした後の試料水に含まれるコイ残存 DNA 量および微生物量を測定した。結果、残存 DNA 量は時間経過に伴って減少し、その減少割合は高温条件でより顕著であったが、環境 DNA 分解に対する微生物量の有意な影響は確認されなかった。これらの結果は、データ解釈および調整におけるこの温度効果の適切な処理が、将来の研究における eDNA 分析の信頼性を増加させることを示唆している。

第三章では、数種(2-4 種程度)を同時に検出するための手法を提案するために、Real-time Multiplex PCR 法を環境 DNA 分析に適用することに取り組んだ。現在、環境 DNA 分析を用いた大型生物の検出には、種特異的 PCR 法およびメタバーコーディング法が主に用いられている。しかし、前者は非標的種 DNA の増幅によるリードの消費により、数種のみが対象とされる場合には費用対効果が低い。一方、後者は対象種ごとに分析を複数回繰り返す必要があるため、より多くの試料と試薬、時間を消費する。そこで、本章では日本産メダカ属 2 種 (*Oryzias latipes, O. sakaizumii*) に種特異的なプライマープローブセットを開発し、Real-time Multiplex PCR 法を用いた複数種の同時検出に供した。結果、水槽実験では、種のバイオマスに偏りがあっても、両種が

全ての試料から検出されることを確認した。さらに、野外調査においても本手法は対象種の生息密度が低い場合を含む全ての調査地点で捕獲調査と一致する結果を示した。Real-time Multiplex PCR 法は他の生物種を対象とした場合にも容易に適用が可能であり、複数の対象種を検出するためのより費用対効果の高い調査を実現する。したがって、今後本手法が共生関係をもつ複数種の検出や、核 DNA とミトコンドリア DNA の比の変化に基づく産卵行動のモニタリングといった調査にとって有用なツールとなることが期待される。

第四章および五章では、環境 DNA 分析の適用可能性を広げる応用技術として、ハイスループットシーケンサー(HTS)を用い、アユ集団の遺伝的多様性を推定することに成功した。さらに、環境 DNA 分析を用いた遺伝的多様性推定における最大の課題であった PCR およびシーケンス時のエラーに由来する無数の偽陽性ハプロタイプを除去するための分析技術を提案した。

第四章では、Amplicon sequence variant (ASV) 法を用いたデノイズ処理と複数のライブラリ反復間における検出率に基づく配列選択を組み合わせた分析技術を提案した。実験では計 9 つの既知ハプロタイプを含む水槽水を試料として用い、15 個のライブラリを調整した。全てのライブラリを HTS によって解析し、検出された真のハプロタイプおよび偽陽性ハプロタイプの数を ASV 法によるデノイズ処理の有無間で比較した。その結果、ASV 法の使用は偽陽性ハプロタイプの数を 5,692 個から 31 個へ大幅に減少させるとともに、8/9 個の真のハプロタイプを検出した。また、検出された真のハプロタイプは全てのライブラリ反復から検出された一方で、偽陽性ハプロタイプの検出率は大幅にばらついていた。したがって、全てのライブラリから検出されたハプロタイプのみを選択することにより、偽陽性ハプロタイプの数は 31 個から 7 個にまでさらに減少した。この研究で提案された分析技術は、環境 DNA 試料から得られた HTS データにおける偽陽性ハプロタイプの大部分を排除することに成功し、環境 DNA 分析を用いた遺伝的多様性推定の検出精度を改善することを可能にした。

第五章では、野外の河川において一般手法で得られたハプロタイプと同一のものを、第四章で提案された分析技術を用いた環境 DNA 分析でも検出できるかどうかを調べ、遺伝的多様性を推定するための環境 DNA 分析の検出力を検討した。実験では、同日に河川のほぼ同地点で水試料とアユ個体を採取した。また、水試料は 20 枚のフィルターを用いて 500mL ずつろ過し、それぞれから得られた環境 DNA 試料について 1st PCR を各 5 反復、2nd PCR を各 3 反復行い、希少なハプロタイプの採水時の補足ミスおよび PCR での増幅ミスの可能性についても検討を行った。一般手法により 96 個体から得られた 42 個のハプロタイプのうち、37 個のハプロタイプが計 20 枚のフィルター反復から検出された。また、捕獲された 96 尾のうち保有していた個体数が多いハプロタイプほど、フィルターおよびライブラリ反復間での検出率が高かった。また、環境 DNA 分析で検出されるハプロタイプのうち一般手法でも得られたものの累積曲線の増加は、各 5 回のライブラリ反復を有する 3 枚のフィルターを分析すると緩やかになった。したがって、環境 DNA 分析は本研究

より反復回数を減少させても遺伝的多様性を推定するための十分な検出力を持っていることが示唆された。環境 DNA 分析は研究者および管理者にとって野生個体群における遺伝的多様性を推定するための実用的なツールとして大きな可能性を有することが示された。

第六章は結論と題し、本研究で得られた成果をまとめている。本研究では、環境 DNA の分解に関する基礎知識を拡充し、結果を踏まえたうえで環境 DNA 分析の適用性を広げる新しい分析手法を提供した。環境 DNA 分解についての知識を深めることは、採取した水試料中に含まれる環境 DNA を確実に検出するために重要である。そのため、新たな研究課題のための手法を開発する際には、この知識を考慮に入れて開発を進めることにより結果の信頼性を向上させることができるだろう。本研究で新たに提案した Real-time Multiplex PCR 法を用いた複数種同時検出法および個体群内の遺伝的多様性を推定するための分析技術は環境 DNA 研究全体の発展に大きく貢献すると期待される。

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Chapter I

General Introduction

1. Introduction

The term of 'environmental DNA (eDNA)' originates in microbiology (Ogram et al. 1987), and it generally means DNA extracted from environmental samples (such as soil, water, and air). Microbiologists have been used eDNA to reveal the microbial world. Since the early study about the use of eDNA for detecting American bullfrog (*Rana catesbeiana*) is reported by Ficetola et al. (2008), there has been an ever-increasing interest to develop and use of eDNA analysis to detect macroorganisms. Especially, in the study of detection of macroorganisms using eDNA analysis, the eDNA means DNA genetic substances contained in environment in a state of feces, saliva, urine and skin cells (Rees et al. 2014a), because an entire organism is almost always not even present in a sample. The application of eDNA analysis for detecting macroorganisms allows us to explore the ecosystem without isolating macroorganisms. Therefore, for ‘macrobial’ ecologists previously reliant on manual count data obtained capture and visual survey, eDNA analysis has become an attractive choice as potential new avenue for the examining the ecosystem (Thomsen & Willerslev 2015). Hence, the published papers about the detection of macroorganisms using eDNA analysis continue to increase at a rapid rate in recent years (Fig. 1–1), and they targeted a wide variety of taxa and habitats (Fig. 1–2, Handley 2015). Especially, aquatic macroorganisms, which inhabit aquatic environment, have been the focus of the studies. The targeted taxa include fishes (e.g., Dejean et al. 2011, Evans et al. 2016, Jerde et al. 2011, Kelly et al. 2014, Laramie et al. 2015), amphibians (e.g., Ficetola et al. 2008, Goldberg et al. 2011, Dejean et al. 2012, Pilliod et al. 2013), crustaceans (e.g., Tréguier et al. 2014, Carim et al. 2016, Forsström and Vasemägi 2016), molluscs (e.g., Goldberg et al. 2013, Deiner and Altermatt 2014, Egan et al. 2015), marine mammals (e.g., Foote et al. 2012), aquatic insects (e.g., Deiner et al. 2015, Doi et al. 2017a) and others.

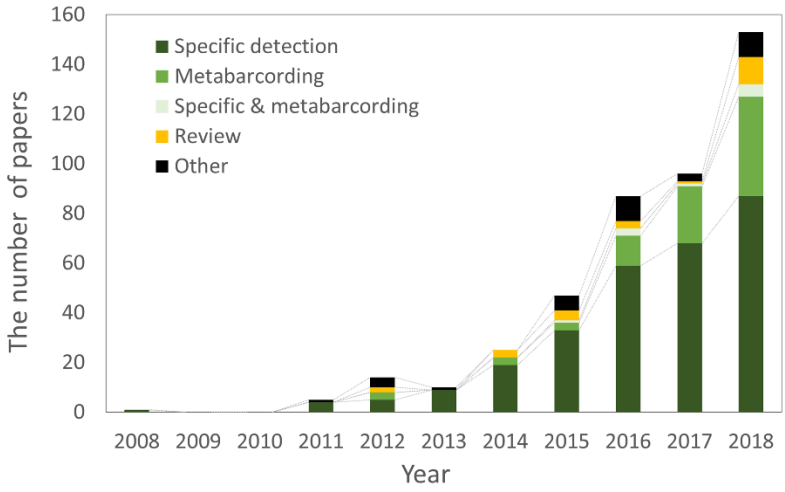

Fig. 1–1 The relationship between the number of papers published in international journals (without pre-printed paper) and published year. The number of papers in 2018 contains published and in press papers by Dec 31, 2018.

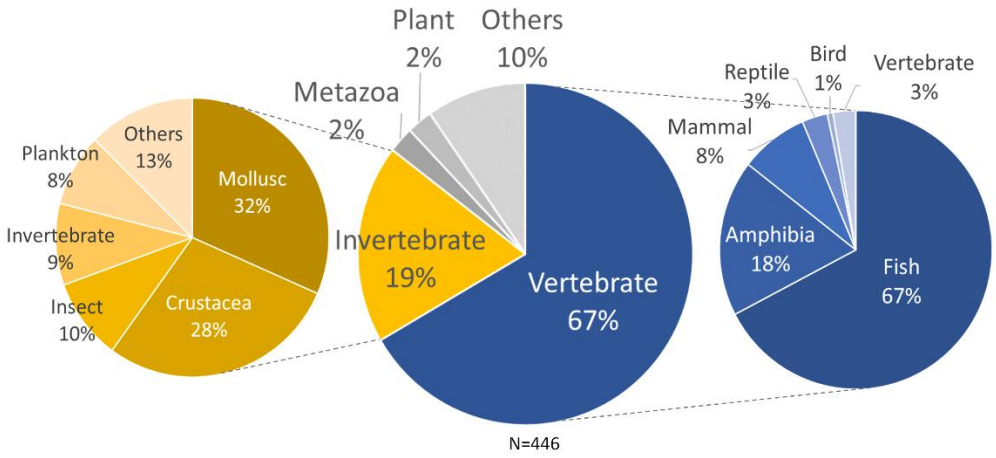

Fig. 1–2 The rates of target taxa in eDNA studies. The pie charts were counted in identifiable minimum group because some studies used taxa specific primer.

Environmental DNA analysis to detect aquatic macroorganisms from water samples involves three basic steps: 1) eDNA collection from water sample, 2) DNA extraction and purification, and 3)

DNA detection. Currently, there are various protocols for eDNA detection of aquatic macroorganisms across various taxa and environments because multiple independent research groups have uniquely developed eDNA analysis techniques (Goldberg et al. 2016). This is characteristic of emerging scientific fields, and it may cause confusion of users. The goal of this chapter is to facilitate a fundamental understanding of major methods in each analysis step by organizing the methods used in each analysis step based on frequency of use and summarizing the characteristics of them. In addition, to develop the efficient biomonitoring method based on eDNA analysis, it is expected that this review could provide information on current status and future challenges in methodological aspects of eDNA analysis. In this chapter, almost all published studies that uses eDNA analysis to detect aquatic macroorganisms were reviewed to calculate the frequency of use of each method in each analysis step. The information of all papers used for calculating the frequencies of the use of each method in each of the analysis steps was listed in Table S1–1.

2. Collection of eDNA in water sample

To collect eDNA from water sample, following three methods has been used: 1) filtration method, 2) ethanol precipitation method, 3) centrifuge and ultrafiltration (Fig. 1–3). The filtration method is the most commonly used method for collecting eDNA in water sample (Fig. 1–3). Each method has a technical advantage and disadvantage, and it is necessary to select which method is better to use depending on the purpose of study and the condition of sample water. Efficient collection of eDNA lead to improvement of detection rate of target species.

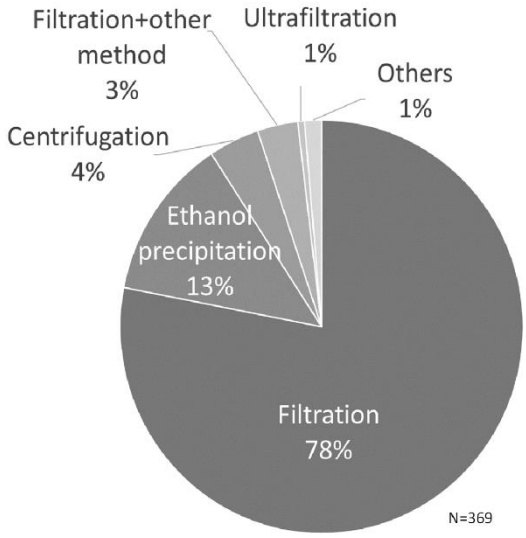

Fig. 1–3 The rates of used method to collect eDNA from water sample.

2.1 Filtration method

The filtration method is the most commonly used method for enriching eDNA in water sample (Fig. 1–3). As the filtration can process larger volumes of water than the other methods, it is the most promising way to obtain higher eDNA yield. Recent studies indicated that the pore size and material of filter can strongly affect the collection of eDNA (Minamoto et al. 2015, Eichmiller et al. 2016) and the majority of macro-organism eDNA seems to be captured by pore size 1–10 μm (Turner et al. 2014). The use of several types of filters has been reported in previous studies, and filters with 0.45 μm (cellulose nitrate), 0.7μm (glass microfiber), and 1.2 μm (glass microfiber) pore size (typical retention diameter) are used in various studies across various taxa and aquatic environments (Fig. 1–4). However, these studies suggested that the type of filters should be carefully selected depending on the properties of the water sample (e.g. aquarium water, lotic system, or lentic system) and the purpose of the study (e.g. species detection, eDNA quantification, or eDNA metabarcoding). Thus, it is desirable to examine a suitable filter for each study in advance. Additionally, when large suspended solids (e.g. algae, or mud) are included in sample water, filter

paper with small pore size easily be clogged. To address this issue, in some studies, pre-filtration using filter with large pore size, coffee filter, and gauze was performed as the preprocessing of collection step (Takahara et al. 2012, Ma et al. 2016, Stewart et al. 2017, Majaneva et al. 2018, Wilson et al. 2014). The pre-filtration can increase processible water volume, but the optimal pore size of pre-filter has been unknown. On the other hand, the use of disposable filter holder (Goldberg et al. 2011, Pilliod et al. 2014, Thomas et al. 2018) or filter capsule with a built-in filter (Miya et al. 2015, Valentini et al. 2016, Civade et al. 2016) are useful way to eliminate the labor associated with bleaching of filtration equipment and the risks of contamination. As a filter capsule, sterivex-GP capsule filters (polyethersulfone 0.22 µm pore size with luer-lock outlet, Millipore, MA) has been the most commonly used, and Spens et al. (2016) demonstrate effectiveness of sterivex-GP for capturing eDNA. In addition, the preserve of sterivex-GP capsule filter by ethanol or Longmire's buffer allows us to store it at room temperature for at least 2 weeks. Therefore, it is suggested that sterivex-GP capsule filter is useful for field survey in remote and harsh conditions.

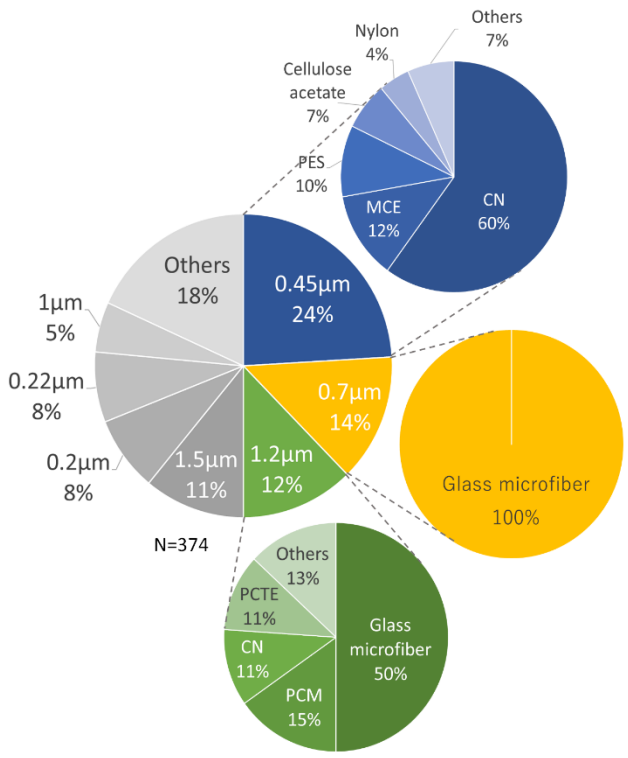

Fig. 1–4 The rates of pore size and material of filters used for filtration in eDNA collection step.

Abbreviations of each filter material are as follows; cellulose nitrate (CN), mixed cellulose ester membrane (MCE), polyether sulfone (PES), polycarbonate membrane (PCM), and polycarbonate track etched (PCTE).

2.2 Ethanol precipitation method

Ethanol precipitation is used when eDNA is collected from small volume (e.g. 15 mL) of water sample (e.g. Deiner et al. 2015, Doi et al. 2015b, Fujiwara et al. 2016). This method is useful for studies at high mountain or rainforest where are difficult to access and to be supplied with electricity, because it requires fewer equipment than other methods and eDNA in water sample can be fixed immediately. However, the detection power in ethanol precipitation is essentially restricted by the limitation of the maximum sample volume due to the necessity of adding twice volume of ethanol (Minamoto et al. 2015). Thus, the ethanol precipitation is suitable only when the eDNA concentration of the target species is high (Doi et al. 2015b). As an alternative option, the isopropanol precipitation was proposed in Doi et al. (2017b) because it is able to increase the processable volume of sample water in reaction volume, since lower volumes of isopropanol are required for precipitation than those of ethanol. They found that isopropanol precipitation recovered twice as much eDNA as ethanol precipitation from both of mesocosm and field samples, when the reaction volumes were equal.

2.3 Centrifuge and ultrafiltration

In centrifuge and ultrafiltration, researchers can collect eDNA from water sample without adding reagents (e.g. Klymus et al. 2015, Takahara et al. 2012). Although these methods are used less often than other methods, the simplicity of the assay gives researchers an advantage when many samples are analyzed. On the other hand, as a common disadvantage of both methods, the maximum sample volume is restricted by the size of centrifuge; thus, it is difficult to analyze large volume of sample

water at once. To address this issue, Doi et al. (2015) combined the filtration method with the ultrafiltration method, eDNA collected on the filter was resuspend in ultrapure water, and then collected eDNA by ultrafiltration. Future studies should test for collection yield of eDNA and usefulness of centrifuge and ultrafiltration.

3 DNA extraction and purification

The DNA extraction and purification method for eDNA analysis is not standardized, and a recent study indicated that the choice of protocols for the extraction of eDNA from filter disk can strongly affect detection of eDNA (Deiner et al. 2015, Minamoto et al. 2015). To extract and purify eDNA which is collected by filtration or other methods, following two methods has been used mainly: 1) commercial DNA extraction kit, or 2) liquid phase separation method.

3.1 commercial DNA extraction kit

The various types of DNA extraction kit are commercially available, and the kind of extraction kit used is different among researchers and/or the purpose of study. For example, DNeasy Blood and Tissue DNA extraction kit (Qiagen, Hilden, Germany) is the most frequently used (Fig. 1–5, Goldberg et al. 2016). In Deiner et al. (2015), the combination of filtration (0.7μm, GF/F) and DNeasy Blood and Tissue DNA extraction kit had the highest overall detection rate, and all target species (*Daphnia longispina, Unio tumidus, Gammarus pulex,* and *Baetis buceratus*) were detected only from this combination. In Eichmiller et al. (2016), they compared the concentration of extracted eDNA and extraction efficiency which were obtained using different kind of six commercial DNA extraction kits; PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA), PowerWater DNA Isolation Kit (MoBio), FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA), FastDNA SPIN Kit for Soil (MP Biomedicals), DNeasy Blood and Tissue DNA extraction kit, and QIAamp DNA Stool Mini Kit (Qiagen). As a result, FastDNA SPIN Kit yielded the highest

concentration of eDNA of carp and was the most sensitive for detection purposes. Furthermore, when much of inhibitors are contained in sample water, the PowerSoil DNA Isolation Kit has been consistently produced extracted DNA that is free of inhibitors (Dineen et al. 2010, Mahmoudi et al. 2011, Smith et al. 2012, Eichmiller et al. 2015). As shown in previous studies, the performance of each commercial DNA extraction kit is dependent on the combination with eDNA collection method and the condition of sample water. Therefore, it is recommended that future eDNA studies take this into consideration and choose DNA extraction kit carefully which is appropriate for each study.

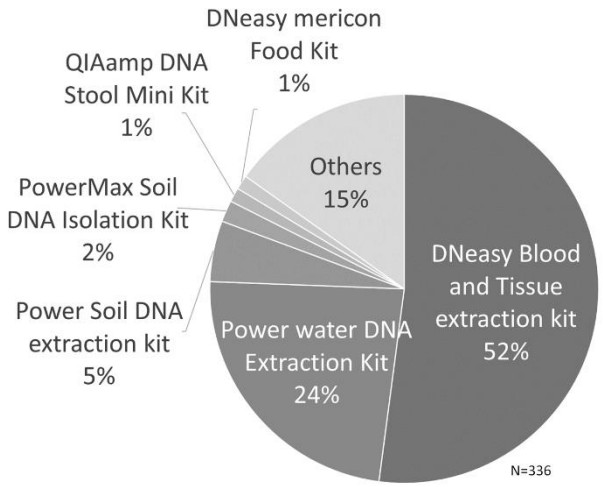

Fig. 1–5 The rates of commercial DNA extraction kit used for DNA extraction.

3.2 liquid phase separation method

In liquid phase separation methods using organic solvent, 1) CTAB (cetyltrimethylammonium bromide) method and 2) PCI (phenol–chloroform–isoamyl alcohol) method have been used mainly (e.g. Turner et al. 2014, Minamoto et al. 2016, Farrington et al. 2015). There are few studies which examined the extraction efficiency; however, in Turner et al. (2014), it showed that the CTAB yielded significantly more eDNA with polycarbonate track-etched filter than the PowerWater DNA Isolation Kit with glass fiber filter (1.5 mm pore size, grade 934-AH). Additionally, in some studies,

it is showed that PCI yield more DNA from glass microfiber filter (GF/F) and cellulose nitrate filter than DNeasy Blood and Tissue DNA extraction kit (Renshaw et al. 2014, Deiner et al. 2015). However, it should be noted that PCI requires careful handling of reagents and the proper waste disposal because it needs to use deleterious substance (phenol, chloroform).

4 DNA detection

The detection of aquatic macroorganisms using eDNA analysis can be divided into following two main types (Thomsen et al. 2015, Takahara et al. 2016, Tsuji et al. 2018): 1) species-specific detection, and 2) eDNA metabarcoding.

4.1 Species-specific detection

The species-specific detection is based on the amplification and detection of short fragments of target species DNA (typically 80–250 bp) by polymerase chain reaction (PCR) with species-specific primers (Bohmann et al. 2014). As a genetic marker, the mitochondrial DNA (mtDNA) is used in most of studies, and the following five regions has been chosen mainly: cytochrome *b* (Cyt*b*), cytochrome c oxidase subunit 1 (COI), 12S ribosomal RNA (12S), 16S ribosomal RNA (16S), and D-loop (Fig. 1–6). Additionally, although there are few studies, it is suggested that nuclear DNA is also sensitive genetic marker for species identification in eDNA studies (Bylemans et al. 2016, Erickson et al. 2016, Minamoto et al. 2017). The species-specific primer is carefully designed by manually or available software such as Primer 3 (Rozen & Skaletsky 2000), Primer Express (Gene Codes Corporation, Ann Arbor, MI, USA) or ecoPCR (Ficetola et al. 2010). Increment of the number of mismatches at near 3'-ends between the target and non-target templates contributes to higher target specificity (Wilcox et al. 2013). Designed primers are generally tested the specificity by *in silico* and/or *in vivo*. For *in silico* test, the sequence similarity assessment program such as primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) that searches the sequence

possible to be amplify by PCR with designed primer set has been generally used. In order to design species-specific primers, every effort should be required to ensure the specificity of primers.

DNA of target species is detected by mainly following three methods in species-specific detection: 1) PCR and gel electrophoresis, 2) real-time PCR, and 3) digital PCR. The main differences between these methods are quantitative capability of DNA and running costs. Thus, eDNA detection method should be selected appropriately according to the necessity of DNA quantification and the research budget.

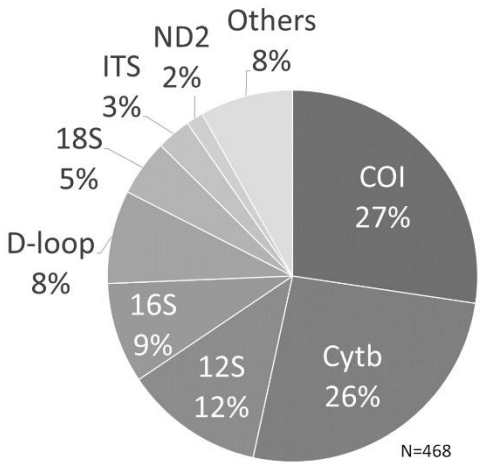

Fig. 1–6 The rates of target regions used in eDNA studies.

4.1.1 PCR and electrophoresis

This is the most classical analysis method, and it can reduce the cost of analysis compared with the other methods. Gel electrophoresis is used to visualize any resulting PCR product, and appearance of PCR gel bands of the correct length indicates positive detection (Evans et al. 2018). DNA concentration can be estimated semi-quantitatively based on the color density of PCR gel band or the number of positives out of PCR replicates (e.g. Doi et al. 2017a, Tsuji et al. 2018). On the other hand, much more time and labor are required for analysis, and multiple extra bands or unclear bands are sometimes produced, leading to unreliable results.

4.1.2 Real-time quantitative PCR (qPCR)

Real-time qPCR has high specificity, sensitivity and quantification ability (Wilcox et al. 2013, Díaz-Ferguson et al. 2014, Turner et al. 2014). DNA concentration is semi-quantitatively or quantitatively estimated based on the number of positives out of PCR replicates or a standard curve obtained using a known concentration of DNA. Especially, probe-based qPCR (for example, TaqMan probe-based) is currently the most efficient tool for eDNA detection of single or few target species (Thomsen et al. 2015). Although the dye-based qPCR (for example, SYBR green) detect any double-strand DNA present in the sample non-specifically, the use of probe-based qPCR allows more specific detection and quantification because probe could be designed to anneal specifically to target region. Hence, real-time qPCR has become the mainstream method for species-specific detection in eDNA analysis in recent years.

4.1.3 Digital PCR

Digital PCR provides sensitive and absolute DNA quantification of target DNA without the use of standard (Doi et al. 2015b, Nathan et al. 2014). In addition, digital PCR has some advantages overcoming the real-time PCR such as the higher tolerance against PCR inhibitors and the lower possibility of the smaller variations in estimates (Doi et al. 2015b). On the other hand, it is important to note that the two disadvantages; researchers cannot confirm 1) the possibility of non-target DNA amplification by sequencing because of the PCR products are unrecoverable, and 2) the problem occurring during PCR cycles because digital PCR measures the end-point of PCR amplification. In addition, digital PCR requires expensive apparatus and higher running costs comparing to real-time PCR. Thus, the effort and development to reduce costs would be needed to digital PCR become a standard method to quantify eDNA concentration in eDNA studies.

4.2 eDNA metabarcoding

In recent years, eDNA metabarcoding using high-throughput sequencing (HTS) has been increased for monitoring aquatic macroorganism assemblages with broader taxonomic scopes (Fig. 1–6). In eDNA metabarcoding, universal primers that amplify a short fragment containing sufficient sequence variation to correctly assign species are designed on homologous genes (i.e., barcode gene regions) of the entire assemblage or community of interest (e.g. all fishes in a stream, or all fishes and amphibians in a pond) (Taberlet et al. 2012, Miya et al. 2015). Following universal amplification of the target barcoding region from eDNA containing a variety of taxa, the amplified DNA fragments are sequenced using HTS, and then the each resulting DNA sequence is assigned to a known taxon by bioinformatic analysis. In bioinformatic analysis, bioinformatic tools such as QIIME (Caporaso et al. 2010), MOTHUR (Schloss et al. 2009), and USEARCH (Edgar 2010) are commonly used, and it typically require the expertise of a bioinformatician proficient in computer programming. However, in eDNA metabarcoding for fish, with the expansion of needs for eDNA metabarcoding, a new user-friendly pipeline has been freely available online in recent years so that HTS data can be analyzed even when expert is absent (e.g. MiFish Pipeline, Sato et al. 2018). Under ideal conditions, it would be possible to assign all OTUs to the species level; however, the level of taxonomic specificity obtained from the bioinformatic assignment is different depending on species-specificity of variations on the marker fragment and the completeness of the reference database (Evans et al. 2018). Thus, in some cases, it should be noted that OTU might be assigned to a family or genus level. More extensive and detailed reviews of eDNA metabarcoding are provided by Deiner et al. (2017).

5 Analytical problems affecting the results of eDNA analysis and countermeasures

In performing eDNA analysis, some analytical problems may lead to an erroneous result. This

chapter focused on the following aspects: 1) DNA contamination, 2) PCR inhibition, and 3) eDNA degradation. The understanding about each problem and the implementation of appropriate measures for them will increase the reliability of the result of eDNA study.

5.1. DNA contamination

All analysis steps potentially have the risk of DNA contamination, and contaminated sample may lead to false positive results. Thus, researchers need to pay sufficient attention to minimize the risk of DNA contamination. In the eDNA collection step, decontamination is generally performed by exposing all equipment (e.g. water sampling cup, sampling bottle, filter holder, and tweezers) to 10% chlorine bleach (sodium hypochlorite, NaClO) for more than ten minutes. Ideally, the risk of contamination will be most significantly reduced by using single-use supplies (e.g. Wilcox et al. 2016). In addition, to confirm that DNA contamination did not occur during eDNA collection step, it is also important to take a cooler blank or field control which is of clean water processed in the same manner with the real samples (Goldberg et al. 2016). In the DNA extraction step, laboratory and equipment should be decontaminated before and after work by using 10% chlorine bleach, DNA contaminate removal solution, or autoclave. Furthermore, the use of a clean bench, positive air pressure, and air filtration will further reduce the risk of contamination. The DNA amplification/sequencing step potentially has highest contamination risk. High-concentration of amplified DNA can easily contaminate the entire laboratory. Therefore, a strict clean-lab protocol that is physically divide rooms for pre- and post-DNA amplification work will significantly limit the contamination risks (Champlot et al. 2010, Willerslev and Cooper 2005).

5.2. PCR inhibition

Humic acids or humic substances, co-extracted with DNA in environmental samples, inhibit amplification of DNA by PCR and cause fail or delay of amplification of target species' DNA (Matheson et al. 2010, McKee et al. 2015). Especially, in quantitative PCR by real-time PCR, PCR

inhibition may cause the misestimation of DNA concentration. As one method for identifying PCR inhibition, researchers add the number of known foreign target DNA copies of foreign DNA to all PCR reactions and compare Ct values (the number of cycles required for enough amplified PCR product to accumulate that it surpasses a threshold recognized by the real-time PCR instrumentation) with that of control sample (Jane et al. 2015, Doi et al. 2017a, Katano et al. 2017, Wu et al. 2018). The presence of PCR inhibitors will shift (delay) the Ct for a given quantity of template DNA, and more than three cycles Ct shift is considered to be evidence of inhibition (Hartman et al. 2005). Additionally, to mitigate the influence of PCR inhibition, the use of bovine serum albumin or inhibitor-resistant polymerases have been reported in some studies (e.g. Barnes et al. 2014, Eichmiller et al. 2014, Deiner et al. 2015, Jane et al. 2015). Furthermore, it is shown that the dilution of eDNA sample is effective for mitigating the effects of PCR inhibition (e.g. McKee et al. 2015, Takahara et al. 2015). However, reduction of the amount of DNA template will face a trade-off between avoiding inhibition and maintaining a detectable concentration of the target DNA in a PCR (cf. Biggs et al. 2015). Thus, dilution rate should be carefully considered because the dilution of eDNA sample decrease the concentration of target DNA.

5.3. eDNA degradation

The eDNA in the water sample is degraded over time (Dejean et al. 2011, Thomsen et al. 2012b, Barnes et al. 2014). Therefore, the eDNA in the collected water sample should be rapidly fixed or collected. On the other hand, when it is difficult to fix or collect of eDNA at the study site, sample water is refrigerated or frozen and transported to laboratory; however, Takahara et al. (2015) suggested that the detection ability in eDNA analysis may be decreased by freezing of sample water. In addition, it has been suggested that the eDNA degradation rate is affected by environmental conditions such as water temperature, bacterial abundance, and ultraviolet (Eichmiller et al. 2016, Pilliod et al. 2014, Stricklar et al. 2015). experimental conditions and environments which were examined previously studies were limited, it is desirable to obtain more information by further

research.

6 Conclusions and perspectives of eDNA analysis

The loss of species diversity in aquatic environments is in a particularly critical situation, and numerous species are endangered (Sala et al. 2000; Dudgeon et al. 2006). Information of species distribution and biomass are critical to ecological management and conservation. Environmental DNA analysis is becoming increasingly accepted and widely used as a useful and efficient tool to collect this information. The technique of eDNA analysis have been examined by many studies and have greatly advanced in the past decade. Currently, various methods are used at each analysis step, since there is no standard protocol. However, it was found that the frequencies of the use of these methods are biased to one or two methods in any analysis step. Comprehending major methods and its future challenges in each analysis step will help researchers, who have been newly engaged in eDNA study, to understand the outline of eDNA analysis. In addition, future challenges for establishing efficient methods in each analysis step were organized by comprehending current status of the methods of eDNA analysis. This information will be helpful in improving the methodological aspect of eDNA analysis in future studies.

7 Overall objectives of this study

Environmental DNA analysis has been used for various research challenges, and its usefulness and availability are demonstrated. On the other hand, there is a concern that confusion and decrease of reliability of results may occur by lack of basic knowledge related to eDNA. Therefore, in future studies, it is desirable to advance the accumulation of basic knowledge and the development of new method in parallel. Thus, the overall objectives of the studies in this theis were to 1) accumulate the basic knowledge about eDNA degradation that affect the results of eDNA analysis and 2) develop the new methods to broaden the applicability of eDNA analysis. As a whole, filter filtration (glass

microfiber with 0.7 μm typical retention diameter) and DNeasy Blood and Tissue extraction kit which were most major method were used for eDNA collection and extraction step. In addition, fish was targeted throughout this study because they have been most used in eDNA study.

As mentioned in this chapter, eDNA degradation is one of the factors that lead to the underestimation of eDNA concentration. In some previous studies, it was suggested that the degradation rate of eDNA is sensitive to local environmental conditions. However, there are still some uncertainties because most previous studies used artificial water and/or examined the effect about limited environmental conditions. Thus, in Chapter II, the relationship between the eDNA degradation rate and these environmental conditions was eamined by focusing on water temperature and bacterial abundance. The accumulation of the basic knowledge about eDNA degradation will increase the reliability of eDNA analysis.

Environmental DNA analysis is a developing technique, and it has the potential to apply for more wide variety of studies. By exploring the potential applicability of eDNA analysis, eDNA analysis wold be used in wider ranges of studies. In Chapter III, a simultaneous detection method of several target species using real-time multiplex PCR method was proposed to offer a more effective option for detecting several species. Currently, metabarcoding and species-specific PCR are being used for eDNA analysis to detect target species. However, metabarcoding is not cost-effective when only a few species are targeted because only the sequence reads of the target species will be analyzed and all the other data obtained by metabarcoding will be left without use. On the other hand, species-specific PCR has to be repeated multiple times, resulting in a consumption of more DNA templates and experimental consumables. Thus, muptiplex PCR would be a suitable method for detecting several species at the same time. Therefore, the determination of the viability of multiplex PCR method in eDNA analysis for the simultaneous detection of several species may broaden the applicability of eDNA analysis.

In Chapter IV and V, a new research framework was proposed for evaluating intraspecific genetic diversity of fish population based on eDNA analysis. Intraspecific diversity is an important component of biodiversity and it affects ecological and evolutionary processes. Thus, monitoring the intraspecific diversity of population is essential to conserve and manage species. However, estimation of the intraspecific diversity using conventional methods based on collection of tissue sample is generally laborious and invasive. On the other hand, because eDNA analysis using HTS allows us to exhaustive sequencing without tissue collecting, it has a potential to resolve issues of conventional methods and provide a new practical way. Although two relating studies were previously reported, it remains a challenge for future studies to develop the method for eliminating erroneous sequences derived from PCR and sequencing errors in HTS data. The achievement of this challenge is necessary to increase the estimation accuracy of intraspecific diversiy by eDNA analysis, because the erroneous sequences are detected as false positive haplotype without proper elimination. Finally, in Chapter VI, the findings of each study in all the other chapters were summed up to conclude the studies in this thesis. I expect that these studies will contribute to the optimization of eDNA analysis and expand the availability of eDNA analysis.

8. Supporting information

Table S1–1. The information of all papers used for calculating the frequencies of the use of each method in each of the analysis steps.

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Chapter II

Water temperature-dependent degradation of environmental DNA and its relation to bacterial abundance

1. Introduction

Environmental DNA (eDNA) analysis has been rapidly developing as a new tool for the biomonitoring of macroorganisms (Ficetola et al. 2008, Jerde et al. 2011, Lodge et al. 2012). The shorter time requirements and cheaper costs of eDNA analysis enable researcheres to conduct long-term and large-scale observations more easily. Sigsgaard et al. (2014) showed that the cost of the eDNA survey for detecting the nearly-extinct European weather loach (*Misgurnus fossilis*) in Denmark was almost half of the prospective cost for a conventional survey when they included the investigators' salaries. Moreover, the time required for the eDNA survey was explicitly shorter than that required for a conventional survey. In addition, they achieved their goals and finally caught the loach with increased efforts, motivated by the detection of eDNA for this species at a specific location from a prior survey. Its outstanding detection capability, in addition to the easy on-site sampling and cost-effectiveness, makes eDNA analysis a prospective tool for natural resource management and ecological studies of biological communities (Fukumoto et al. 2015, Yamamoto et al. 2016). In addition, eDNA analysis has been applied not only for detection of species, but also for biomass estimations, as eDNA concentrations are positively correlated with biomass or abundance (Takahara et al. 2012, Doi et al. 2016, Yamamoto et al. 2016).

However, there are still some uncertainties about eDNA dynamics that could potentially result in significant errors regarding eDNA quantification. For example, it has been reported that eDNA is degraded rapidly over time after being released into the water from organisms (Thomsen et al. 2012, Maruyama et al. 2014) and that degradation rates were reported to have considerably large variation

among studies (Eichmiller et al. 2016). Variation in eDNA degradation rates can be caused by variation in local environmental conditions, such as water temperature, pH, and light intensity (Barnes et al. 2014, Pilliod et al. 2014, Strickler et al. 2015). Previous studies indicate that detailed knowledge of eDNA degradation is essential for improving estimations of initial eDNA concentrations at the sampling point and, therrby, improbing estimations of biomass.

Thomsen et al. (2012) developed an eDNA degradation model based on time-dependent changes in the eDNA concentrations in sea water of the European flounder (*Platichthys flesus*) and the three-spined stickleback (*Gasterosteus aculeatus*). Many studies adopted the time-dependent exponential decay of eDNA as the model of degradation (Maruyama et al. 2014, Pilliod et al. 2014, Strickler et al. 2015, Eichmiller et al. 2016); however, by only observing the time-dependent degradation of eDNA, their constructed models only incorporated time as a variable, without determining the effects of other environmental factors on degradation rates. It has been suggested that eDNA degradation is decelerated at low water temperatures due to lower bacterial activities (Eichmiller et al. 2016, Pilliod et al. 2014, Stricklar et al. 2015). This observation suggests potential effects of bacterial abundance on the degradation rate of eDNA and its dependence on temperature.

In most previous studies, tank experiments using well water (Barnes et al. 2014), store-bought spring water (Pilliod et al. 2014), or tap water (Maruyama et al. 2014) were performed to determine the degradation rates of eDNA derived from individual target fish that were put into the aquariums. Although these experiments using artificial water provided helpful insight into the mechanisms of the eDNA degradation process, the properties of the water (dissolved organic compounds, suspended solids, and microbial abundance) were different from those of natural aquatic ecosystems. To the best of our knowledge, there have been only two reports that estimated eDNA degradation rates using field water (Thomsen et al. 2012, Eichmiller et al. 2016). Although the degradation rates could vary among habitats depending on water quality, microbial abundance, and water temperature,

information on rates is currently available on from sea water and lake water samples (Thomsen et al. 2012, Eichmiller et al. 2016); additional information on degradation rates in different habitats would be desirable. Moreover, clarification on the relationship among water temperature, bacterial abundance, and degradation rates of eDNA would promote a better understanding of the effects of water quality on eDNA degradation.

The purpose of this study was to determine the water temperature-dependent degradation rate of eDNA shed by ayu (*Plecoglossus altivelis altivelis*) and common carp (*Cyprinus carpio*) by using water samples from a river inhabited by both species and to construct a refined nonlinear model that additionally incorporates the effect of water temperature in the existing degradation model. This would be a more versatile model that could estimate the initial concentration of eDNA at the sampling time when we need to correct for the degradation of eDNA during transportation of the water samples. The laboratory experiments were performed using purified common carp DNA to determine the effects of bacterial abundance, water temperature, and the interactions of these two factors on the degradation of eDNA. Understanding these two related factors of eDNA degradation would help to elucidate eDNA dynamics under field conditions.

2. Materials and methods

2.1. Experimental design

In the present study, two experiments were performed. In Experiment 1, time and water temperature dependent degradation of eDNA in field water was monitored using concentration measurements of eDNA in water samples incubated at three different temperatures. In Experiment 2, the relationship between bacterial abundance and the eDNA degradation rate was examined using aquarium water as a source of bacteria and purified DNA of common carp as the eDNA source at three temperatures. To keep maintain relatively constant initial eDNA concentration across experimental replications, a

known amount of purified common carp DNA was added to each water sample that was collected from an aquarium without common carp. The field water was not used because the water of Yasu River originally contained the eDNA of the common carp and would have produced a large unintended variation in eDNA concentrations among replications due to the heterogeneous distribution of eDNA in the field water. In this experiment, bacterial abundance would have been better controlled independently of temperature or time to determine its effect on eDNA degradation directly, i.e., spiking different amounts of bacteria into water samples to vary bacterial abundance in controls. However, the initial concentration of bacteria in the aquarium water was used for the incubation experiment due to difficulties in separating and concentration total bacteria from water. The methods described in the next two sections (Water filtration and DNA collection; Real-time quantitative PCR) were the same for both experiments. The temperature of the thermostatic water bath was kept constant during each experiment.

2.2. Water filtration and DNA collection

All water samples were filtered using a Whatman GF/F filter (GE Healthcare Life Sciences, Piscataway, NJ, USA), with a diameter of 47 mm and a nominal pore size of 0.7 μm. Each filter disk was folded inward in half with tweezers and wrapped in aluminum foil, then placed in a plastic bag with a zipper and stored at ±20°C until DNA extractions were performed. To prevent contamination between samples, all filtration instruments were cleaned before use by immersing in 10% bleach solution for 5 min, washing with running tap water, and then rinsing with Milli-Q water. Further, to monitor contamination from experimental equipment, Milli-Q water was filtered at the same volume as sample water at each sampling time point during the incubation experiment. Identical experimental steps were applied to both experimental samples and the Milli-Q water treatment.

DNA was extracted and purified using the protocol described by Tsuji et al. (2016). Each half folded frozen filter was rolled into a cylindrical shape without unfolding and placed in the upper

part of the spin column with 2.0-mL collection tubes (EZ-10, BioBasic, Ontario, Canada). Silica gel membranes, equipped to the EZ-10 spin column, were removed and discarded prior to use. Reagents from the DNeasy Blood and Tissue Kit (Qiagen, Manufacturer Location) were used with the EZ-10 spin columns (Bio Basic) for DNA extraction and purification. The spin columns were then centrifuged for 1 min at 6,000 × g to remove any excess water contained in the filter. Four hundred microliters of Milli-Q water, 200 µL of buffer AL, and 20 µL of proteinase K were mixed and dispensed onto the filter in each spin column, and the spin columns were incubated for 15 min at 56EC. After incubation, the spin columns were centrifuged for 1 min at 6,000 × g, and the eluted filtrate was transferred to a new 1.5-mL microtube. Four hundred microliters of Tris-EDTA buffer (pH 8.0) were added to each filter, and the filter was incubated for 1 min at room temperature before being centrifuged for 1 min at 6,000 × g. The upper part of the spin column containing the filter was removed from the 2.0-mL collection tube and the first and second filtrates were combined in the 2.0-mL tube. Then, 200 µL of buffer AL and 610 µL of ethanol were added to the combined filtrates and mixed well by gently pipetting up and down. The DNA in each mixture was purified with the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instruction. Each mixture was transferred to a spin column provided by the DNeasy Blood & Tissue Kit to trap DNA fragments on its silicagel membrane by centrifugation. Because of the large volume of each mixture, this step was repeated three times to catch all the DNA on the membrane. The silica-gel membrane was then washed twice with the washing buffers AW1 and AW2, and DNA was eluted from the column with 100 µL of buffer AE. The DNA extractions from filters were carried out within 24 and 48 hours after the filtration step in Experiments 1 and 2, respectively.

2.3. Quantitative real-time PCR

Environmental DNA was quantified according to the method described by Takahara et al. (2012). Quantitative real-time TaqMan1polymerase chain reaction (PCR) was performed using a StepOne-Plus Real-Time PCR system (Applied Biosystems, FosterCity, CA, USA) to estimate copy

numbers of the target DNA in each sample. A specific region of the eDNA from each target species was amplified by using the previously reported primers and probe sets: PaaCyB-Forward (50-CCTAGTCTCCCTGGCTTTATTCTCT-30), Paa-CyB-Reverse (50-GTAGAATGGCGTAGGCGAAAA-30), and Paa-CyB-Probe (50-FAM-ACTTCACGGCAGCCAACCCCC-TAM RA-30) for the ayu mitochondrial cytochrome b gene (Yamanaka et al. 2016); and CpCyB_496F (50-GGTG GGTTCTCAGTAGACAATGC-30), CpCyB_573R (50-GGCGGCAATAACAAATGGTAGT-30), and CpCyB_550p probe (50-FAM-CACTAACACGATTCTTCGCATTCCACTTCC-TAMRA-30) for the common carp mitochondrial cytochrome *b* gene (Takahara et al. 2012). It was confirmed that the primer and probe set specifically detected DNA of the target species in the surveyed area (Takahara et al. 2012, Yamanaka et al. 2016).

Real-time PCR was performed in triplicate for each eDNA sample, standard dilution series, and PCR negative controls. PCR was conducted in 20-μL volumes and the reagent consisted of 900 nM of each primer, 125 nM of TaqMan1probe, plus sample DNA (the amounts of sample DNA used in each experiment are described below) in 1 × PCR master mix (TaqMan1 Gene Expression Master Mix; Life Technologies, Carlsbad, CA, USA). The artificially-synthesized target sequence from ayu DNA (399 bp) was cloned into qTAKN-2 plasmids, while the target sequence from common carp DNA was cloned into the pGEM plasmid. Both were used as standards for real-time PCR analyses for each species (Takahara et al. 2012, Yamanaka et al. 2016). A standard dilution series containing 3×10^1 to 3×10^4 copies of the target sequences were analyzed in triplicate for each PCR test. For negative controls, instead of adding DNA template, the same volume of Milli-Q water was added to the PCR reactions. The PCR thermal conditions were as follows: 2 min at 50°C, 10 min at 95°C, then 55 cycles of 15 s at 95°C, and 60 s at 60°C. The R^2 values of the standard curve ranged from 0.986 to 0.993 for ayu and from 0.987 to 0.994 for common carp in Experiment 1 and from 0.984 to 0.988 for common carp in Experiment 2.

2.4. Experiment 1: Water temperature-dependent degradation of eDNA

On July 22, 2014, 52 L of water was sampled from the surface of the Yasu River (35°2′23″N, 136 ° 1′19″E, Ritto, Japan) where ayu and common carp were living. The water quality parameters measured were pH 7.54, temperature 26.1°C, and electrical conductivity 0.33 mS/cm, as determined by water quality sensors (HI 98128 pHep 5, HI 98312 DiST 6, and HI 98312 DiST 6, respectively; HANNA Instruments, Woonsocket, RI, USA). The sample waters were immediately transported on ice to the laboratory (amount of time required was 1 h 40 min). In the laboratory, 2 L of sample water was divided into four portions of 0.5 L each and filtered with a GF/F filter as the initial samples, and 0.5 L of Milli-Q water was filtered as an equipment blank. At the same time, the remaining sample water was dispensed into 12 tanks with 4 L of water each, and 4 tanks were assigned to each of three different incubation temperatures (mean ± standard deviation 10 ± 0.6°C, 20 ± 0.1°C, and 30 ± 0.2°C) maintained by a thermostatic water bath. The water temperature of each bath was recorded with a temperature logger during the experiment. After 1, 3, 6, 12, 24, and 48 h from the initiation of incubation, 0.5 L of water from each tank was sampled and filtered with a GF/F filter in the same manner as the initial sample. DNA extractions from the filters and quantification by real-time PCR were performed according to the method described above. The following amounts of extracted DNA were used as DNA template for each reaction in the real-time PCR: 2 μL for the ayu experiment and 5 μL for the common carp experiment.

2.5. Experiment 2: Relationship between eDNA degradation and bacterial abundance

On January 07, 2015, 3 L of surface water were sampled from an aquarium containing two non-target species; goldfish (*Carassius auratus*) and dark chub (*Nipponocypris temminckii*). The water quality parameters measured were pH 7.72, temperature 18.6°C, and electrical conductivity 0.15 mS/cm. The sample water was pre-filtered to retain particle sizes down to 6 μm and to remove large impurities such as residues of food and feces. The filtered water was expected to contain bacteria and was used as medium water in the following experiment. Purified total DNA from

common carp, extracted from skeletal muscle tissue, was then spiked into the sample water and the mixture was stirred; the final DNA concentration was 0.5 ng/ mL. In a subsequent incubation experiment, the concentration of common carp DNA and the bacterial abundance in the sample water were measured, along with the elapsed time. As the initial sample (N_0) at time 0, 100 mL × 4 replications of the sample water were filtered with a GF/F filter. The remaining sample water was divided into 12 bottles with 215 mL in each, and 4 bottles were submerged in each of three thermostatic baths set at 10°C, 20°C, and 30°C. The water temperature in each thermostatic bath was recorded by temperature logger during the experiment. After 12 and 24 h, 100 mL of water from each of the four tanks in each of the three thermostatic baths were filtered with a GF/F filter. As an equipment blank, 100 mL of Milli-Q water was filtered at each time point. DNA extractions from the filters and quantification by real-time PCR were conducted according to the method described above. The following amounts of extracted DNA were used as DNA template for each reaction in the real-time PCR: 1 μL for the initial sample and 9 μL for the 12- and 24-h samples.

In parallel with the filtration, to investigate bacterial abundance in the sample water, microorganisms were cultured using the Standard Method Agar “Nissui” (code: 05618; Nissui Pharmaceutical, Tokyo, Japan) according to the manufacturer's instructions. The composition of the Standard Method Agar per 1 L included: yeast extracts 2.5 g, peptone 5.0 g, glucose 1.0 g, and agar 15 g. The sample water was diluted fivefold with sterilized water. One milliliter of the diluted water sample was added to each of two petri dishes and then approximately 20 mL of the Standard Method Agar was admixed to coagulate. The Congealed Standard Method Agar was incubated for 24 h at 35°C. Afterward, the number of colonies was counted, and the average number of colonies between two replicated petri dishes were calculated as colony forming unit (CFU)/mL for each of six experimental conditions (two time periods [12 and 24 h] × three temperatures [10°C, 20°C, and 30°C]).

2.6. Statistical analysis

All data on DNA concentration and microbial abundance were calculated as number of DNA copies per volume of filtered sample water and were used for statistical analysis. All statistical analyses were performed with R ver. 3.1.0 software (R Core Team 2014).

In Experiment 1, the influence of time and water temperature on eDNA concentration was evaluated using generalized linear mixed-effects models (GLMM, package nlme) with a random effect of individual tanks. The resulting full model formula (shown in the conventional expression in R language) was lm (log [eDNA copies] ~ elapsed time + elapsed time: water temperature). Water temperature was incorporated into the model as an interaction term with time because the initial eDNA copies were the same for each water temperature. The coefficient of the interaction effect of time and water temperature at 10°C was set as 0. The confidence intervals of the coefficient among the three temperature conditions was compared using the glht function implemented in the amultcompo package of R (Hothorn et al. 2008). The minimum level of significance was set at p = 0.05. Then, referring to Thomsen et al. (2012), the time-dependent eDNA decay model was extended to cover the water temperature-dependent effect.

The model is as follows:

$$\frac{dN}{dt} = -(a + bk)N \qquad (1)$$

Solving this gives:

$$N_t = N_0 \exp[-(bk + a)t] \qquad (2)$$

The parameters are as follows: N_t is the DNA concentration at time t (hours). N_0 is the initial DNA concentration, k is the water temperature (°C), t is the time (elapsed time in hours), and a and b are estimated by the nls function in R.

The half-decay time t (hour) was calculated by the following equation:

$$\frac{N_0}{N_t} = \frac{N_0}{N_0 \exp[-(bk + a)t]} = 2 \qquad (3)$$

Solving this gives:

$$t = \ln(2) - (bk + a) \qquad (4)$$

In Experiment 2, the generalized linear model (GLM) of glm function in R was used to evaluate the influence of elapsed time, water temperature, and bacterial abundance on eDNA concentration. GLM was performed on the assumption of normal distribution, the log of common carp DNA concentration was set as the response variable, while the explanatory variables were elapsed time, water temperature, and bacterial abundance. Thus, the full model formula was glm (log [eDNA copies] ~ elapsed time + water temperature + bacterial abundance). GLM analysis was repeated to assess the effects of elapsed time and water temperature on bacterial abundance by setting bacterial abundance as the response variable and elapsed time and water temperature as explanatory variables. The full model formula was glm (bacterial abundance ~ elapsed time + water temperature).

3. Results

In Experiment 1, the eDNA concentrations in water decreased exponentially with elapsed time. The initial eDNA concentrations of ayu and carp in the initial sample water were 229,901 ± 16,763 and 2,558 ± 345 copies/500 mL (mean ± standard deviation), respectively. Environmental DNA degraded faster at a higher water temperature in both fish species (Fig. 2–1, Table S2–1). The coefficients of the interaction term of water temperature and time were different with all combinations of the three temperature controls for both ayu and common carp ($p < 0.01$), except for temperatures between 20°C and 30°C in common carp ($p = 0.07$); there were stronger negative values at higher water temperatures (Table 2–1). The resulting nonlinear model fitted for ayu and common carp is shown in Fig. 2–1 (the equations of the full models are shown in Table 2–2). DNA degraded faster at a more higher water temperatures for both species, so the half-decay times were shorter at higher water temperatures (Table 2–2). The constants a and b were estimated as $a = -0.07081$ and $b = 0.01062$ for ayu and $a = -0.07372$ and $b = 0.01075$ for common carp.

In Experiment 2, the relationship between the eDNA concentrations of common carp and bacterial abundances in bottles after 12 and 24 h of incubation was evaluated (Fig 2–2, Table S2–2). The

initial sample contained 647,803 ± 88,106 (mean ± standard deviation) copies/100 mL of common carp eDNA and 1,473 ± 188 (mean ± standard deviation) CFU/mL of bacteria (Table S2–2). GLM analyses revealed that elapsed time and water temperature had significant negative effects on the degradation rate of common carp DNA (-5.35×10^{-2} and -1.35×10^{-1}, respectively; $p < 0.001$ for both factors), but there was no significant effect on the carp DNA from bacterial abundance (5.99×10^{-5}; p = 0.097). Elapsed time and water temperature also affected bacterial abundance (-2.57×10^{2} and 5.05×10^{2}, respectively; $p < 0.001$ for both factors). Ayu and common carp DNAs were not detected in any equipment blanks or PCR negative controls in either experiment, indicating no cross-contamination during sample processing.

Table2–1. Results of generalized linear mixed-effects model analysis in Experiment 1 showing the effects of time and water temperature on eDNA concentration. All explanatory variables were significant at $p = 0.05$. The differences among coefficients of the interaction terms of elapsed time and water temperature were compared using the 95% confidence interval for the coefficients. The same superscript letters associated with the coefficients indicate statistical equivalence at $p = 0.05$ in each species. Note that the difference between 20°C and 30°C was marginally significant for common carp ($p = 0.07$).

response variable	explanatory variable	coefficient	standard error
log (ayu sweetfish eDNA)			
	elapsed time (h)	–0.024	0.004
	elapsed time (h): water temperature 10˚C	0 [a]	
	elapsed time (h): water temperature 20˚C	–0.058 [b]	0.005
	elapsed time (h): water temperature 30˚C	–0.091 [c]	0.005
	Intercept	12.024	0.06
log (common carp eDNA)			
	elapsed time (h)	–0.027	0.005
	elapsed time (h): water temperature 10˚C	0 [a]	
	elapsed time (h): water temperature 20˚C	–0.045 [b]	0.006
	elapsed time (h): water temperature 30˚C	–0.079 [b]	0.008
	Intercept	7.533	0.07

Table 2–2. Full models and half-decay times for time-dependent degradation of eDNA as a result of nonlinear model fitting in Experiment 1.

species	full model	water temperature (˚C)	half-decay time (h)
Ayu sweetfish	$N_t = 229,901.2 \times exp[-(0.01062 \times k - 0.07081) \times t]$		
		10	19.55
		20	4.89
		30	2.80
Common carp	$N_t = 2,558.0 \times exp[-(0.01075 \times k - 0.07372) \times t]$		
		10	20.50
		20	4.90
		30	2.80

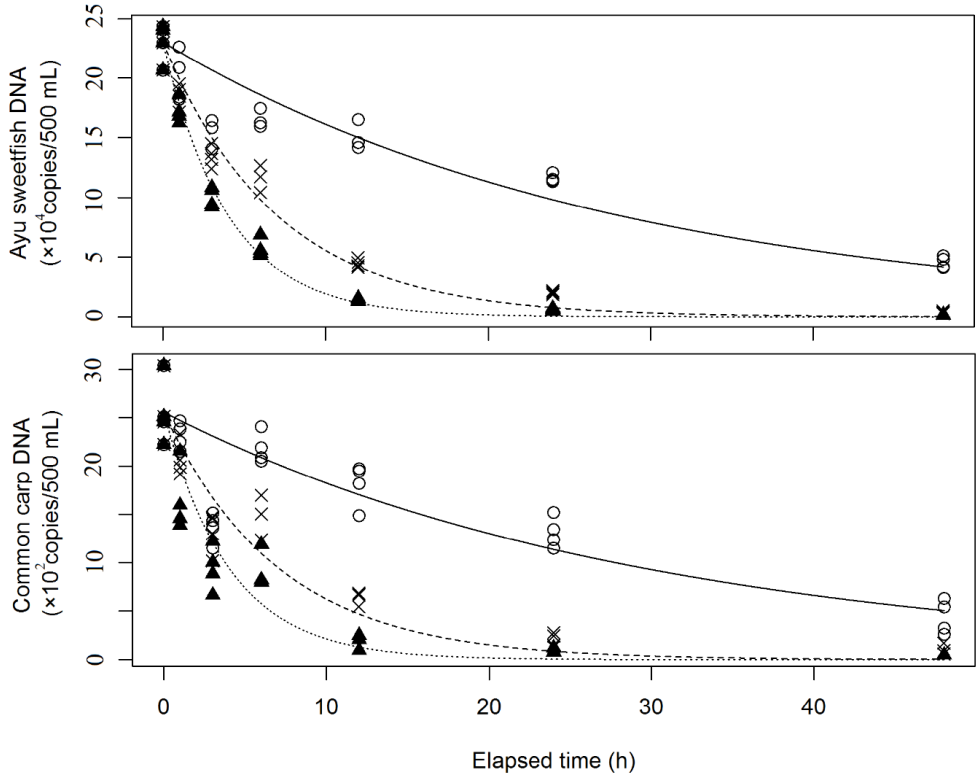

Fig. 2–1. Time-dependent changes in eDNA concentration for ayu and common carp. Circles, crosses, and triangles represent DNA concentrations for each species at 10°C, 20°C, and 30°C, respectively. Solid, dashed, and dotted lines represent nonlinear regression for 10°C, 20°C, and 30°C treatments, respectively.

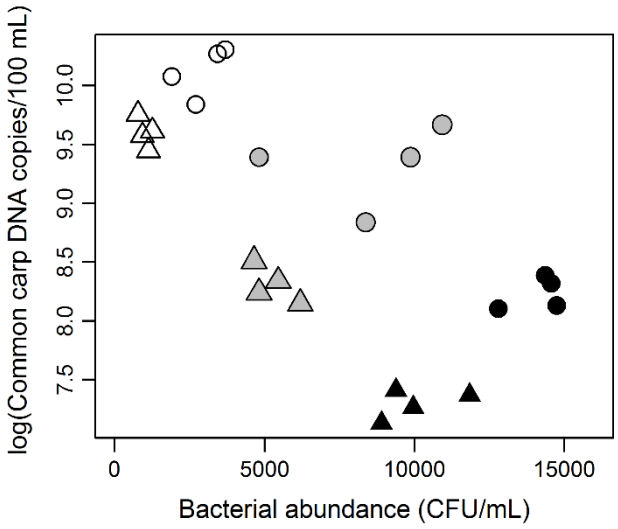

Fig. 2–2. Relationship between bacterial abundance and DNA concentration of common carp in water after 12 and 24 h of incubation. Data for 12- and 24-h incubation trials are represented as circles and triangles, respectively. White, gray, and black plots represent water temperature settings of 10°C, 20°C, and 30°C, respectively.

4. Discussion

The present study showed that eDNA of ayu and common carp in sample water degraded rapidly with time and that the degradation rate was affected by water temperature. Environmental DNA degraded faster at higher water temperatures, which is consistent with previous studies (Stricklar et al. 2015, Eichmiller et al. 2016). The strong effect of water temperature on degradation rates suggests the importance of controlled storage temperatures during transportation; keeping water samples cool during transportation would retard the degradation of eDNA. To the best of our knowledge, an eDNA degradation model that explicitly incorporates water temperature has not been previously reported. To detect any differences among sites for sampling time or water temperature, it would be better to estimate initial eDNA concentrations in sample water at the time of collection. At this point, concentrations can be used for comparing biomass among sampling sites, which requires highly accurate estimations. The temperature-dependency of the eDNA degradation should also be considered when interpreting eDNA concentrations in the field to estimate target species

biomass in different seasons or different times of day.

Water temperature-dependent degradation of eDNA in Experiment 1 led us to speculate that the degradation of eDNA was strongly influenced by the activity of bacterially-secreted DNA-degrading enzymes. However, in Experiment 2, it was found that bacterial abundance did not have a significant effect on the degradation of eDNA, whereas elapsed time and water temperature did have significant effects on the eDNA degradation rates (Fig. 2–2). In this study, one of the simplest traditional methods was used, i.e., standard culturing method, to estimate bacterial abundance, although this estimate does not include bacteria that are not amenable to culture. Other alternative methods such as flow cytometry or qPCR would provide a more comprehensive estimate for bacterial abundance; however, the culture-based method can provide a reliable index of bacterial abundance if the ratio of culturable bacteria to total bacteria has not changed among samples. Bacterial abundance and water temperature were highly correlated, i.e., bacterial abundance was higher at higher water temperatures. Ideally, the bacterial abundance and temperature should be independently controlled to explicitly determine their effects on the eDNA degradation; however, controlling bacterial abundances in field samples is complicated and multi-faceted. If it is possible to inject known amounts of bacteria, it could have separated the effects of bacterial abundance and water temperature on the degradation rate. Furthermore, a condition-dependent relationship is likely to exist between bacterial abundance and eDNA concentration; a positive correlation between bacterial abundance and eDNA concentration appeared when a single temperature treatment (for example 30°C) was focuesd, whereas a negative correlation appeared when three temperature treatments were considered altogether (Fig. 2–2). Many complex processes would be involved in eDNA degradation such as the dynamics of bacterial populations, the production of DNA-degrading enzymes by bacteria, and the enzymatic degradation rates of eDNA; therefore, bacterial populations can influence eDNA concentrations directly or indirectly. These complex mechanisms would be a source of the condition-dependent relationship between eDNA concentration and bacterial

abundance. Additionally, eDNA in field water exists in various states such as free-floating DNA and DNA contained in organelles and cells (Turner et al. 2014, Tsuji et al. 2016). If most of the eDNA is contained in organelles and cells, as suggested by Turner et al. (2014) and Tsuji et al. (2016), it is plausible that the bacterial effects would be slower and the rate of eDNA degradation would be slower. It would be ideal to use eDNA samples reflecting the realistic components of eDNA in natural samples; however, purified DNA was used instead in Experiment 2 because it was difficult to isolate eDNA components from field water without bacterial contamination. Further studies on the effects of bacteria on eDNA degradation would be desirable, paying attention to the state of the eDNA and the condition-dependent effect of bacteria.

Eichmiller et al. (2016) reported the degradation constant k, equivalent to β (/h) in Thomsen et al. (2012), for common carp eDNA at four different water temperatures using a simple degradation model reported by Thomsen et al. (2012). The constants were 0.015 ± 0.00083, 0.078 ± 0.0046, 0.10 ± 0.0063, and 0.10 ± 0.0063 (average ± standard error, /h) at 5°C, 15°C, 25°C, and 35°C, respectively. In the degradation model constructed in this study, the degradation rate was denoted as $bk + a$ (/h), which includes the effect of water temperature and is equivalent to k in Eichmiller et al. (2016) when the water temperature factor (k) is substituted. As a result, the degradation rate at each temperature can be calculated as −0.020, 0.088, 0.20, and 0.30/h at 5°C, 15°C, 25°C, and 35°C, respectively. Compared with the rates of Eichmiller et al. (2016), the estimated degradation rates in this study were higher, at 15°C, 25°C, and 35°C (especially at 35°C, with a rate three times that in the previous study). Suspended humic materials and clays can slow down DNA degradation due to their binding effect on DNA fragments, resulting in protection from enzymatic degradation (Stotzky et al. 2000). In addition, the lower and higher degradation rates at lower and higher water temperature conditions, respectively, seem to be mainly due to the adoption of the linear function to approximate the effect of water temperature. It might be unreasonable to incorporate the linear function to approximate the water temperature effect; however, the present model would not lose its practicability since the extremely warm condition in which the degradation constant was

overestimated is not commonly observed in the temperate zone. For example, the temperature range of the surface water in the Yasu River, Shiga, Japan, is 4°C to 28°C (Endoh et al. 2007). Knowledge of the effects of water qualities (temperature, pH, conductivity) on eDNA degradation compared to those in field water, such as suspended humic materials, suspended solids, and microbial abundance is required for better estimates of macro species distribution and biomass based on eDNA concentrations (Yamamoto et al. 2016). The present study showed that the eDNA degradation rate is strongly influenced by water temperature and we cannot ignore this effect in efforts to improve the accuracy of quantification of eDNA. Because the degradation of eDNA is rapid and sensitive to environmental factors, further clarification of the relationship between eDNA degradation and other environmental factors will increase the reliability of biomass estimations based on eDNA analysis.

5. Supplementary information

Table S2-1. All raw data obtained in Experiment 1. The eDNA concentrations of ayu and common carp at each water temperature and elapsed time.

Table S2-2. All raw data obtained in Experiment 2. The eDNA concentrations of common carp and bacterial abundance at each water temperature and elapsed time.

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Chapter III

Real-time multiplex PCR for simultaneous detection of multiple species from environmental DNA: an application on two Japanese medaka species

1. Introduction

The information on species distributions is crucial not only for basic ecological research but also for population management and conservation. Various surveillance methods such as baited traps, cast nets, and visual observation has conventionally been used for researches on aquatic organisms (Lodge et al. 2012, Minamoto et al. 2012); however, the information obtained from these methods is likely to vary in quality for at least three reasons. First, capture efficiency or discovery rate depends on the researcher's skill. Second, substantial effort might be needed to find rare species4, and this effort can degrade the reliability of "absence" data. Finally, species identification on the basis of morphology usually requires taxonomic expertise (Keskin et al. 2016). These characteristics of traditional methods will obstruct standardized surveys, particularly large-scale surveys. Furthermore, capture surveys might be unfavorable for endangered species because the sampling activities can damage target-species populations or their habitats. In addition, these traditional methods require a lot of time and labor.

To overcome these obstacles, eDNA has recently been used (Ficetola et al. 2008, Goldberg et al. 2011). An extremely promising aspect of eDNA analysis is the ability to use the efforts of people who are not trained in the method used to collect samples (Lodge et al. 2012, Minamoto et al. 2012). Furthermore, eDNA analysis, which relies on genetic information, can readily distinguish species that are morphologically similar to each other (Fukumoto et al. 2015). The simplicity of sampling and a well-established laboratory workflow can provide consistent results and minimize any impacts from sampling activity on target-species populations or their habitats. eDNA analysis is

also relatively cost-effective. For example, the use of eDNA analysis to determine the distribution of the nearly extinct European weather loach (*Misgurnus fossilis*) decreased the prospective cost of a conventional survey by one-half when they included the investigators' salaries (Sigsgaard et al. 2014).

Environmental DNA analysis for detecting aquatic macroorganisms can be divided into two main types, metabarcoding or species-specific PCR (Takahara et al. 2016). The metabarcoding technique, which uses a next-generation sequencer, enables us to obtain DNA sequence data of phylogenetically broad taxa in a single analysis (e.g., all Teleostei) (Miya et al. 2015, Yamamoto et al. 2017); however, when only a few species are targeted, metabarcoding is not efficient because of the amplification of non-target species DNA. Furthermore, metabarcoding can sometimes fail to distinguish closely related species because of the genetic homology on the amplified DNA region. On the other hand, the species-specific PCR technique, which uses primers that amplify only target-species DNA, is suitable for surveys targeting only a subset of a community (e.g., monitoring an endangered species) and for samples including closely related species that cannot be distinguished by the sequence on the amplified DNA region (Ficetola et al. 2008, Jerde et al. 2011). However, when multiple species are targeted, PCR is required for each individual species (Mahon et al. 2014, Pfleger et al. 2016) so that the cost in time or money and the amount of DNA samples would increase depending on the number of target species.

To address these PCR issues, a new methodological framework is suggested for simultaneous detection of a limited number of species by analyzing eDNA using real-time multiplex PCR. Real-time multiplex PCR uses a set of species-specific primers and probe that is labeled with different fluorescent dyes for each target species so that approximately two to five species (depending on the experimental conditions) can be detected simultaneously in a single real-time PCR reaction. Real-time multiplex PCR, as opposed to real-time single PCR, shortens the

processing times and reduces the use of reagents (Saponari et al. 2013, Bernáldez et al. 2014). In this study, this methodological framework was applied for detecting the *Oryzias* species complex in Japan, generally called 'medaka' fish, which includes *O. latipes* and *O. sakaizumii* (hereafter, *latipes* and *sakaizumii*, respectively). The two medaka species are so morphologically similar that reliable species identification cannot be made without DNA analysis (Asai et al. 2011). In addition, they are partially sympatric (Kume et al. 2010), yet their distribution on a local scale remains unknown. Both species are listed as 'Vulnerable' on the *Red List of Threatened Species of Japan*, and are endangered because of the invasion of non-native species and habitat degradation (Hosoya et al. 2000, Ministry of the Environment 2015) The knowledge of species-specific distributions is crucial for their effective management and conservation.

In this study, new species-specific primer probe sets at specific regions of mitochondrial DNA was developed to detect and distinguish the two medaka species in Japan. The specificity of the developed primer probe set for each species was tested using genomic DNA from the respective target species and real-time single PCR. In addition, the developed primer probe sets were tested with respect to whether they could detect only respective target species using real-time multiplex PCR with a mixture of *latipes* and *sakaizumii* genomic DNA. To assess the capability of this detection system, the effect of the biased abundance of the species on the detection capability was examined in aquarium experiment. In addition, the effectiveness of these primer probe sets was tested by determining the distributions of the two medaka species in their known natural habitats.

2. Materials and methods

2.1. Primer probe design

The sequence data of the mitochondrial complete genome for the two species of Japanese medaka (*latipes* and *sakaizumii*) and those of two non-target species [*Gambusia affinis* (mosquitofish) and

Poecilia reticulata (guppy)] both of which are introduced species and have similar habitat preference with medaka were obtained from the National Center for Biotechnology Information database. All sequence data used for designing the primer set of each medaka species are listed in Table S3–1. The *latipes* primers were designed on the ND5 gene and named OlaND5-F/R, and the *sakaizumii* primers were designed on the 16S rRNA gene and named Osa16S-F/R (Table 3–1). The designed primers for each of the two medaka species had the species-specific nucleotide at the 3′ ends. Primer-BLAST with the default settings was used to the primer parameter checks and *in silico* tests. The lengths of the PCR products were 108 and 136 bp for *latipes* and *sakaizumii*, respectively. The TaqMan probes for each species, named OlaND5-Pr for *latipes* and Osa16S-Pr for *sakaizumii* respectively, were designed on the PCR products by each of the species amplification primers (Table 3–1). For simultaneous detection by real-time PCR, the 5′ end of the *latipes* and *sakaizumii* probes were respectively labeled with different fluorescent dyes (Table 3–1).

2.2. Primer probe test with genomic DNA

The specificity of the designed primer probe sets was confirmed by real-time single PCR with extracted genomic DNA from three individuals each *latipes* and *sakaizumii*. Real-time single PCR was performed in a 15-μL reaction mixture for each sample using the StepOnePlus Real-Time System (Life Technologies, Foster City, CA, USA) and analyzed using StepOneSoftware v2.3. The reaction mixture contained a set of the primers OlaND5-F/R or Osa16S-F/R at a final concentration of 900 nM each, 125 nM TaqMan probe, and 0.1 ng genomic DNA (*latipes* or *sakaizumii*) in 1 × TaqMan gene expression Master Mix (Life Technologies, Carlsbad, CA, USA). To assess the occurrence of unintended cross-contamination, PCR negative controls (PCR-NCs) comprising ultrapure water instead of genomic DNA were prepared. The PCR thermal conditions were as follows: 2 min at 50ºC, 10 min at 95ºC, and 55 cycles of 15 s at 95ºC and 60 s at 60ºC. Real-time single PCR for each DNA sample and PCR-NCs were performed in triplicate.

2.3. Real-time multiplex PCR with genomic DNA

The two tests of real-time multiplex PCR using OlaND5-F/R/Pr and Osa16S-F/R/Pr were conducted to examine (i) whether each species could be specifically detected and (ii) whether both species could be simultaneously detected. All real-time multiplex PCRs were performed in a 15-μL reaction mixture for each sample using StepOnePlus Real-Time System and analyzed using StepOneSoftware v2.3. In addition, final concentrations of TaqMan probes in the real-time multiplex PCRs were adjusted to equalize the fluorescence intensities as follows: Osa16S-Pr was set to 41.7 nM (one-third of OlaND5-Pr) because the fluorescence intensity of *sakaizumii* (Rn = 2.99) was approximately three times stronger than that of *latipes* (Rn = 1.04). The reaction mixture contained four amplification primers at a final concentration of 900 nM each, TaqMan probes OlaND5-Pr at a final concentration of 125 nM and Osa16S-Pr at a final concentration of 41.7 nM, and 0.1 ng genomic DNA of one of the four species (*latipes*, *sakaizumii*, mosquitofish or guppy) in 1 × PCR TaqMan gene expression Master Mix. For simultaneous detection test of two medaka species in real-time multiplex PCR, the reaction mixture contained four primers at a final concentration of 900 nM each, TaqMan probes OlaND5-Pr at a final concentration of 125 nM, and Osa16S-Pr at a final concentration of 41.7 nM, and a 0.2 ng mixture of genomic DNA from the two medaka species in 1 × PCR TaqMan gene expression Master Mix. The PCR-NC preparation for both tests used ultrapure water instead of genomic DNA. The thermal condition for the real-time multiplex PCR was same as real-time single PCR. All real-time multiplex PCRs for each DNA sample and PCR-NCs were performed in triplicate. All of the aforementioned real-time PCR complied with the MIQE checklist (Bustin et al. 2009) (Table S3–2).

2.4. Aquarium experiments with biased abundance

To examine the effect of biased abundance of the two medaka species on detection capability, our detection system was applied to the aquariums with varying abundance ratios for each medaka species. Thirteen aquariums (35 × 20 × 21 cm) that contained 6 L aged tap water were prepared and

were aerated throughout the experiment. In 12 of the 13 aquariums, 10 individuals of adult medaka were placed in each aquarium at a varying abundance ratio of *latipes* to *sakaizumii* (1:9, 5:5, and 9:1, with four replications each). An aquarium without a target species was also prepared as the experimental control to check for cross-contamination during the aquarium experiment. The aquariums were kept in the laboratory at 20 ºC room temperature with a 12-h light/dark cycles. After maintaining the fish for 4 days with no food, 50 mL surface water was collected from the center of each aquarium. The collected water samples were immediately filtered through glass fiber filters with a mesh size of 0.7 μm (GF/F, GE Healthcare Japan, Tokyo, Japan). Each filter disc was folded inward in half, wrapped in aluminum foil, placed in a plastic bag with a zipper, and stored at −20ºC until DNA extraction. To avoid contamination, all sampling and filtering equipment were dipped in a 10% bleach solution for >5 min, carefully washed with tap water, and finally rinsed with ultrapure water.

eDNA was extracted from the filter discs following the procedures of Yamanaka *et al.* (2016). Each filter disc was rolled into a cylindrical shape and placed into a spin column (EZ-10 SpinColumn & Collection Tube; Bio Basic Inc., Ontario, Canada), from which a silica-gel membrane was prospectively removed. After removing excess water on the filter by centrifugation, the mixture, containing 200 μL ultrapure water, 100 μL buffer AL, and 10 μL proteinase K, was added to the filter. The spin columns were incubated at 56ºC for 15 min. The spin columns were then centrifuged for 1 min at 6000 × *g*, and upper parts of the spin columns were removed and placed on new 2-mL collection tubes. Then, 200 μL TE buffer (pH 8.0) was added onto the filter, which was incubated for 1 min at room temperature. Spin columns were centrifuged for 1 min at 6000 × *g*, and the elution was mixed with the first filtrate, 200 μL buffer AL and 600 μL 100% ethanol. The mixture was then purified using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. At the final elution step, DNA was eluted from the DNeasy spin column with 100 μL of buffer AE. The buffer AL, buffer AE and proteinase K used for DNA extraction were the DNA attachment reagents from the extraction kit. The extracted eDNA was

amplified by real-time multiplex PCR in the same manner as described above for real-time multiplex PCR with genomic DNA. All reactions were performed on a single run. As a DNA template, each 1 μL DNA sample was added.

2.5. Assay of field-collected samples

A field survey using our detection system was conducted to examine whether the detection system could be applied for practical researches. This survey was conducted at the drainages of the Yura River system in the Tanba and Tajima Districts, Japan. The eight sampling sites (sts. 1–8) selected were the same sites as those from Kume & Hosoya (2010) with new st. 9 added for this study (Fig. 2–1). All water sampling was conducted on October 16, 2016. The 0.5 L of surface water was collected using a plastic cup in each sampling site. To avoid contamination, different equipment was used for each water sampling, and different investigators, respectively, performed water sampling and capture survey. The water quality of each sample is shown in Table 3–4. Collected water samples were filtered immediately using the on-site water filtration system (Yamanaka et al. 2016) with GF/F filters and were immediately kept at −20ºC until use. As an experimental control, the same volume of ultrapure water was filtered and treated in the same manner as that in the samples. DNA was extracted from the filters using the same method as that used in the aquarium experiments. The extracted DNA was amplified by real-time multiplex PCR in the same manner as above for real-time multiplex PCR with genomic DNA. Two microliters of DNA samples were used as the DNA template. The PCR amplified products were commercially sequenced using the Sanger sequencing method.

After water sampling, three investigators conducted the capture survey at sts. 1, 3, 5, 7, 8, and 9 using hand nets. The number of captured individuals was limited to a maximum of 30 fish at each sampling site, and they were carefully handled as much as possible to avoid pain and stress. Captured fish were quickly fixed in 70% ethanol onsite. The catch per unit effort (CPUE) as the

number of fish/net * hour was calculated and used as the index of fish density. In st. 2, Kume & Hosoya (2010) found only *sakaizumii*, but both medaka species were found in a later survey (Iguchi *et al.* unpublished); therefore, the PCR-restriction fragment length polymorphism (PCR-RFLP) analysis on cytochrome *b* genes was performed using captured individuals from sts. 2 and 9 to determine the resident species. For PCR-RFLP analysis, total genomic DNA was extracted from muscle or fin tissues of the 30 captured individuals using conventional phenol-chloroform methods (Asahida et al.1996). For st. 2, the 30 individuals captured on June 20, 2016, were used for PCR-RFLP analysis following Takehana *et al.* (2003). PCR-amplified segments were digested with enzyme *Hae*III, and the fragments were confirmed by electrophoresis on 3% agarose gels, dyed with Midori Green (Nippon Genetics Europe GmbH, Duren, Germany). The two medaka species were distinguished based on the respective diagnostic fragment patterns (pattern A–I for *latipes* and pattern J–H for *sakaizumii*). Interestingly, Kume & Hosoya (2010) found both medaka species in st. 5, but neither species has been found since 2013 because of the subsequent revetment construction (Iguchi *et al*. unpublished).

2.6. PCR inhibition test for field-collected eDNA samples

To evaluate the presence or absence of PCR inhibition, the Ct shift was compared between the field-collected samples and controls (only DNA elution buffer) with the same number of foreign DNA copies. When PCR inhibition was occurred by PCR inhibitors in field-collected sample, Ct for a given quantity of foreign DNA would shift (delay), comparing with the controls. To check whether the field-collected eDNA samples cause the PCR inhibitions or not, known DNA copies of *Trachurus japonicus* (Japanese jack mackerel), a marine fish and does not inhabit in the study sites, were spiked in the PCR reactions with eDNA sample or Buffer AE, respectively. The primer probe set reported in Yamamoto et al. (2016) was used: Tja-CytB-Forward primer, 5′-CAGATATCGCAACCGCCTTT-3′; Tja-CytB-Reverse primer, 5′-CCGATGTGAAGGTAAATGCAAA-3′; Tja-CytB-Probe, 5′-FAM-

TATGCACGCCAACGGCGCCT-TAMRA-3′. Each PCR reaction mixture (15 μL total volume) contained 900 nM of each primer (Tja-CytB-F and -R), 125 nM TaqMan probe (Tja-CytB-Pr), 2 μL of the field-collected DNA sample or Buffer AE, and plasmid DNA containing the cytochrome *b* gene of *T. japonicus* (1.5×10^2 copies) in 1 × PCR TaqMan gene expression Master Mix. The real-time PCR was performed in triplicate. Ct values of the PCR results from the two settings were compared and calculated as $\Delta Ct = Ct_{sample} - Ct_{control}$. When Ct values shift ≥3 cycles, it was considered as the evidence of inhibition (Hartman et al. 2005, Goldberg et al. 2016, Doi et al. 2017, Katano et al. 2017).

3. Results

No DNA was detected in any experimental control or PCR-NCs, confirming the absence of cross-contamination during sample processing. In *in silico* test using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), the designed primer sets for *latipes* and *sakaizumii* were found to amplified only the target medaka species. In real-time single PCR with extracted genomic DNA, both of the developed primer probe sets for *latipes* and *sakaizumii* showed species specific amplification (Table 3–2). In real-time multiplex PCR with genomic DNA extracted from single species (*latipes*, *sakaizumii*, mosquitofish, or guppy), both of developed primer probe sets showed species-specific amplification (Table 3–3). In real-time multiplex PCR with the mixture of extracted genomic DNA from both medaka species, DNA was detected from each (Table 3–3). In this real-time multiplex PCR with adjusted probe concentrations, each TaqMan probe showed approximately equal fluorescence intensity (*latipes* Rn = 0.97, *sakaizumii* Rn = 0.92).

In aquarium experiment examining the effect of biased abundance of the two medaka species on the detection capability, both species were simultaneously detected with 50-mL sampling in all replicates of real-time multiplex PCR even when the abundance of the two species was biased.

In the field survey, the abundance of medaka species varied among sites, with the calculated CPUE ranging from 0.4 (st. 3) to 100 fish/net*h (st. 8; Table 3–4). The distributions of the two medaka species inferred by real-time multiplex PCR of eDNA were consistent with those determined in the previous study (Kume et al. 2010) and the capture survey (Fig. 3–1). In the results of PCR-RFLP analysis, both medaka species were found in st. 2 at a ratio of 1 *latipes* to 29 *sakaizumii*, and only *latipes* was found in st. 9. In the analysis of eDNA from st. 2, *latipes* was detected in two out of three PCR replications, whereas *sakaizumii* was detected in all three replications. In st. 9, only *latipes* was detected in all three PCR replications of eDNA samples. In st. 5, no medaka species were captured, and neither species was detected in the eDNA analysis. In the other sites (sts. 1, 3, 4, 6, 7, and 8), the distributions of the two medaka species inferred by our eDNA analysis were consistent with those in the previous report (Kume et al. 2010). In the eDNA analysis for these sites, all three PCR replications produced consistent results, but for *latipes* from st. 3, which was detected in only one replication. Amplification specificity of the real-time multiplex PCR of all field-collected samples was confirmed by direct amplicon sequencing (Table S3–3). In inhibition test, the ΔCt values from the internal controls of all samples were lower than 3, indicating that the inhibition was absence in field-collected samples (Table S3–4).

Table 3–1. List of designed primers and TaqMan probes. Fluorescence excitation spectra are as follows: JOE 529 nm, FAM 495 nm. Fluorescence emission spectra are as follows: JOE 555 nm, FAM 520 nm.

Target species	Primer name	Sequence (5′–> 3′)
latipes	OlaND5-F	TCTTTACTATAATCCTGGCAGTCCTTATC
	OlaND5-R	CTGCTGCTAACTCTTTTTGTTGTTC
	OlaND5-Pr	[JOE]-AATCTAACTGCTCGCAAAGTCCCACGACT-[BHQ]
		(Amplicon length = 108 bp)
sakaizumii	Osa16S-F	ATCTTCAAGTAGAGGTGACAGACCA
	Osa16S-R	AACTCTCTTGATTTCTAGTCATTTGTGTC
	Osa16S-Pr	[FAM]-TGGATAGAAGTTCAGCCTC-[NFQ]-[MGB]
		(Amplicon length = 136 bp)

Table 3–2. Results of the primer-probe specificity test with genomic DNA using real-time single polymerase chain reaction. The identification numbers of the genomic DNA template indicates different individuals. The repeatability of three PCR replications are shown as SD of Ct values.

Primer probe set	genomic DNA template (0.1 ng)		PCR positive (repeatability)	
			latipes	*sakaizumii*
OlaND5-F, R, Pr (real-time single PCR)	*latipes*	1	3/3 (0.14)	−
		2	3/3 (0.20)	−
		3	3/3 (0.11)	−
	sakaizumii	1	0	−
		2	0	−
		3	0	−
Osa16S-F, R, Pr (real-time single PCR)	*latipes*	1	−	0
		2	−	0
		3	−	0
	sakaizumii	1	−	3/3 (0.19)
		2	−	3/3 (0.03)
		3	−	3/3 (0.13)

Table 3–3. Results of the primer-probe specificity test with genomic DNA using real-time multiplex polymerase chain reaction. The identification number of the genomic DNA template indicates different individual. The repeatability of three PCR replications are shown as SD of Ct values.

Primer probe set	genomic DNA template (0.1 ng)		PCR positive (repeatability)	
			latipes	*sakaizumii*
Both (real-time multiplex PCR)	*latipes*	1	3/3 (0.11)	0
		2	3/3 (0.11)	0
		3	3/3 (0.17)	0
	sakaizumii	1	0	3/3 (0.10)
		2	0	3/3 (0.07)
		3	0	3/3 (0.44)
	latipes 1 and *sakaizumii* 1		3/3 (0.19)	3/3 (0.14)
	latipes 2 and *sakaizumii* 2		3/3 (0.09)	3/3 (0.01)
	latipes 3 and *sakaizumii* 3		3/3 (0.22)	3/3 (0.12)
	mosquitofish		0	0
	guppy		0	0

Table 3–4. Summary of water quality data, the calculated catch per unit effort, results of reaction polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), and reported inhabiting species by Kume & Hosoya (2010) at each sampling site.

St.No.	Water temperature (ºC)	pH	Electrical conductivity (mS/cm)	CPUE (fish/net*h)	PCR-RFLP		Reported inhabiting species
					latipes	*sakaizumii*	
1	18.0	7.87	0.30	3.7	–	–	*sakaizumii*
2	17.5	8.58	0.35	–	1	29	*sakaizumii*
3	19.2	8.02	0.35	0.4	–	–	both
4	23.2	10.49	0.18	–	–	–	*sakaizumii*
5	22.9	7.99	0.21	–	–	–	both
6	24.7	8.55	0.20	–	–	–	*latipes*
7	24.9	8.02	0.28	20	–	–	*sakaizumii*
8	23.9	7.95	0.14	100	–	–	*latipes*
9	22.3	7.99	0.17	20	30	0	–

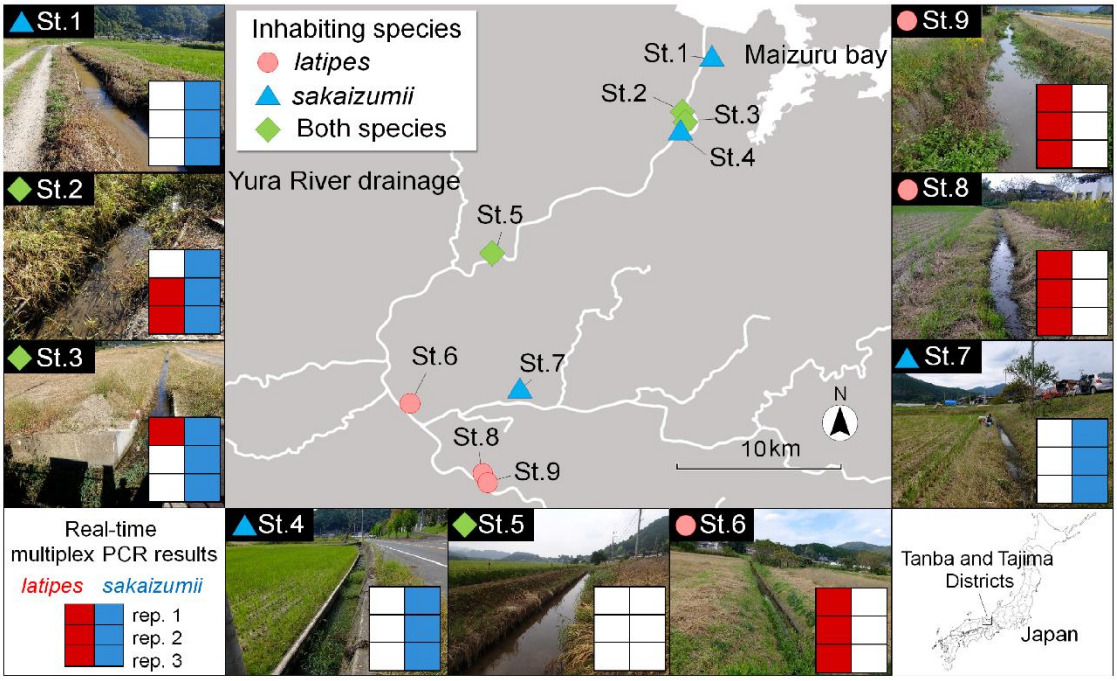

Fig. 3–1. Distribution of the two medaka species determined by the environmental DNA survey with real-time multiplex polymerase chain reaction (PCR) and the capture survey. Results of the capture survey are consolidated data of PCR-restriction fragment length polymorphism

analysis in our study and Kume and Hosoya (2010). Marks on each site indicate inhabiting species determined by capture survey (circle; *Oryzias latipes*, triangle; *O. sakaizumii*, diamond; both species). Closed boxes of red (*O. latipes*) and blue (*O. sakaizumii*) indicate the positive results of real-time multiplex PCR with three replications.

4. Discussion

The new analytical technique that using eDNA analysis with real-time multiplex PCR was developed and applied to a two medaka species system in this study. The developed primer probe sets for each medaka species could amplify species specific genomic DNA in both real-time single and multiplex PCR (Table 3–2, 3–3). When eDNA from the aquariums was analyzed, biased abundance of the two medaka species in an aquarium did not prevent detection of both species in any real-time multiplex PCR with sufficient sample volume. When this detection system was applied in the field survey, the distributions of medaka species inferred by eDNA were consistent with those of capture survey results, including sites with low fish density (Table 3–4, Fig. 3–1). Thus, our detection system for the two medaka species is reliable and useful for determining their distributions, and in general, eDNA analysis with real-time multiplex PCR would be applicable to other aquatic systems. Real-time multiplex PCR has two advantages over conventional real-time single PCR. First, whereas single PCR requires an amplification reaction for each single target species, real-time multiplex PCR can simultaneously detect a few target species in a single reaction, thereby reducing reagent use, labor, and time. This cost-efficiency makes it possible to increase the numbers of target species and samples. Second, real-time multiplex PCR can reduce the use of PCR samples. The remaining samples can be stored and used for further analyses (Takahara et al. 2015).

In the aquarium experiment, the less abundant species were detected in all real-time multiplex PCRs even when the abundance between the two medaka species was biased toward one side. Consistent with the aquarium experiment, the less abundant species were successfully detected in the field

surveys (*latipes* in sts. 2 and 3). In both sts. 2 and 3 of the field survey, the fish densities of *latipes* were much lower than that of *sakaizumii*. In st. 2, the ratio of the captured individuals of *latipes* to *sakaizumii* was 1:29. In st. 3, CPUE was smallest in all sampling sites, and the relative ratio of the *latipes* to *sakaizumii* was reported to be 1:4 (Kume et al.2010). These results suggest that *latipes* is scarcely distributed in these sites. The eDNA concentration increases with the abundance and/or biomass of organisms (Takahara et al .2015, Pilliod et al. 2013, Doi et al. 2016, Yamamoto et al. 2016); therefore, the eDNA concentrations of *latipes* in sts. 2 and 3 were most likely low. Less abundance of eDNA generally raises the probability of PCR dropout (i.e., failure in PCR amplification). One or two positives out of three PCR replicates for *latipes* in sts. 2 and 3 might be caused by less abundance of the species in those sites. Multiple PCR replicates appear to reduce false negatives, even in species with a low density, and are possibly also used as a proxy for biomass as suggested by previous studies on the positive relationship between biomass and the number of positives in the PCR replicates (Doi et al. 2017).

Our field survey confirmed a new sympatric habitat (st. 2) and the recent disappearance of both medaka species (st. 5). There is a high possibility that the previous study in st. 2 (Kume et al. 2010) failed to find *latipes* because of the insufficient number of individuals sampled (n = 10) given that our result indicated that *latipes* is much less abundant than *sakaizumii* (1 vs. 29) in this site. In addition, there is another possibility that *latipes* might have dispersed into this site after the previous study because st. 2 is located downstream of st. 3, where *latipes* inhabits, and the sites are connected to each other by a runnel (the distance between two sites is ~ 560 m). In any case, non-invasive sampling and the high reliability of eDNA analysis can facilitate long-term sustainable surveys, especially for endangered species, such as the two medaka species. The absence of both medaka species in st. 5 indicated by the result of both eDNA analysis and the capture survey suggests the recent extinction of them after the previous survey (Kume et al. 2010). In st. 5, lining of channels was changed to three-sides concrete after the previous capture survey (2010). This

human activity might cause habitat manipulation because the medaka species are vulnerable to anthropogenic habitat disturbances (Takehana et al. 2010, Mamun et al. 2016). Our eDNA analysis with high detection capability can be an effective tool for tracing a species habitat change.

Application of real-time multiplex PCR to other systems requires some technical considerations. First, primer probe sets must be highly species specific to accurately detect only the target species. TaqMan probe chemistry was adopted to improve the specificity of the analysis. Second, each primer probe set must have approximately the same melting temperature (Tm) because any difference in Tm might cause DNA amplification bias. Third, uncompetitive primers and probes must be designed and used for the reactions. When more species are targeted in a single reaction, the possibilities of primer (probe) dimer generation and probe fluorescent competition will be increased. Finally, the length of target sequences must be similar among the target species because the amplification efficiency and the eDNA decay rate in ambient water should be affected by the length of the DNA fragment (Jo et al. 2017).

The present study has demonstrated that multiple species can be simultaneously detected using real-time multiplex PCR from eDNA although only a few targeted species were included. This eDNA analysis with real-time multiplex PCR should be useful for large-scale and long-term distribution surveys that focus on a few multiple species and could be easily applied to various combinations of species. Real-time multiplex PCR will be more cost-effective than the conventional single PCR method. This cost effectiveness allows the scale-up of distribution surveys with increasing target species and/or sampling sites. eDNA analysis with real-time multiplex PCR will allow us to conduct more effective surveys and monitoring of natural communities without the time and budget constraints, and that will contribute toward our understanding and the conservation of biodiversity.

5. Supplementary information

Table S3–1. A list of all sequence data used for designing the primer set of each medaka species.

Table S3–2. MIQE Guidelines Checklist.

Table S3–3. The sequence data that were obtained from amplicons of field samples.

Table S3–4. Results of inhibition test using Trachurus japonicus DNA as internal positive control.

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Chapter IV

Evaluating intraspecific diversity of a fish population using environmental DNA: An approach to distinguish true haplotypes from erroneous sequences

1. Introduction

Genetic diversity is a key component of biodiversity and is necessary for species' adaptation to changing natural and human-induced selective pressures (Allendorf et al. 2012, Laikre et al. 2016). Intraspecific diversity of a fish population has typically been analyzed by capturing individuals using traditional capture methods, such as baited traps, casting nets, electrofishing among other methods, followed by genetic analysis of each individual. However, these methods have at least two major limitations: 1) use of traditional sampling approaches causes damage to the target organisms, and 2) large sampling efforts are necessary to provide an accurate estimate of intraspecific diversity across an entire population. Traditional capture methods may threaten the persistence of species/population, particularly for rare and endangered species. In addition, insufficient sampling might lead to an underestimation of intraspecific diversity of a population (Xing et al. 2013). These limitations may reduce the feasibility of surveys and increase the uncertainty of results.

Environmental DNA analysis has recently been used to detect the distribution of macroorganisms, particularly those living in aquatic habitats (Ficetola et al. 2008, Lodge et al. 2012, Thomsen et al. 2015). Environmental DNA analysis allows for non-invasive and cost-effective detection of the presence of a species in a habitat because only water samples are needed to analyze instead of capturing and/or observing the target species (Thomsen et al. 2015). Because of this advantage and its high sensitivity, eDNA analysis has frequently been applied for the detection of not only common species but also rare and endangered species (Fukumoto et al. 2015, Ishige et al. 2017, Katano et al. 2017, Rees et al. 2014b). In addition, an approach involving eDNA metabarcoding using

high-throughput sequencing (HTS) can effectively and comprehensively reveal the aquatic community structure, and thus, it has been gaining attention as a powerful tool for biodiversity monitoring (Kelly et al. 2014, Miya et al. 2015, Yamamoto et al. 2017).

Current applications of eDNA analysis have been limited mostly to the detection and identification of species (e.g. Rees et al. 2015, Thomsen et al. 2015). However, eDNA analysis potentially can be extended to evaluate of intraspecific diversity, because eDNA released from multiple individuals coexist in a water sample. Recently, Uchii et al. (2016 and 2017) have developed a method using cycling probe technology and real-time PCR to quantify the relative proportion of two different genotypes of common carp (*Cyprinus carpio*) based on a single nucleotide polymorphism (SNP). These studies revealed that the SNP genotypes were 'embedded' in eDNA samples suspended in the field water. Furthermore, Sigsgaard et al. (2016) applied eDNA analysis for estimates of whale shark (*Rhincodon typus*) intraspecific diversity and found multiple haplotypes that had been identified previously from tissue-derived DNA by Sanger sequencing. These findings show the power and effectiveness of eDNA analysis for analyzing intraspecific diversity of target species. However, caution should be exercised during the use of HTS for intraspecific diversity, because HTS data usually include many erroneous sequences that are generated during PCR and sequencing (Coissac et al. 2012, Edgar et al. 2016, Schloss et al. 2011).

Researchers have tried to address the issue of erroneous sequences using multiple approaches, including the use of high-fidelity DNA polymerase in PCR, quality filtering based on base-call scores and/or clustering of sequences into operational taxonomic units (OTUs, OTU methods). The use of high-fidelity DNA polymerase in PCR contributes to decreased sequencing errors in PCR products (Ramachandran et al. 2011), but it is not completely prevented. The OTU methods involve clustering of sequences that are more different from each other than a fixed dissimilarity threshold (typically 3%; Callahan et al. 2016, Hughes et al. 2017). Thus, true haplotypes that are similar to each other are

clustered into single OTU, leading to incorrect evaluations of intraspecific diversity. Therefore, OTU methods cannot be applied to analyze intraspecific diversity. To evaluate intraspecific diversity using eDNA samples, it is necessary to develop effective novel approaches to eliminate erroneous sequences inherent in HTS.

Intraspecific diversity of a population might be analyzed more effectively by use of amplicon sequence variant (ASV) methods, which have recently been developed in the fields of microbiology for correcting erroneous sequences derived from HTS data (e.g. Callahan et al. 2017 and references therein). ASV methods infer unique biological variants in the sample without imposing the arbitrary dissimilarity thresholds that define OTUs. As a core process of an ASV method, 'denoising' is performed using an error model that assumes the biological sequences are more likely to be observed than erroneous sequences (e.g., DADA2; Callahan et al. 2016). The sensitivity and accuracy of ASV methods with respect to correcting erroneous sequences have been shown to be better than those of OTU methods (Callahan et al. 2016, Edgar et al. 2016, Eren et al. 2013, Eren et al. 2015, Needham et al. 2017). The high resolution of biological sequences afforded by ASV methods has the potential to improve the accuracy of evaluating intraspecific diversity inferred from eDNA.

The purpose of this study is to propose an approach for eliminating false positive haplotypes derived from erroneous sequences in HTS data obtained from an eDNA sample and demonstrate the usefulness of eDNA analysis for the evaluation of intraspecific diversity of a fish population. In this study, genetic diversity in the Ayu (*Plecoglossus altivelis altivelis*) fish was examined, an important fisheries target in Japanese inland waters whose genetic diversity has been evaluated in previous studies (e.g. Iguchi et al. 2002, Takeshima et al. 2016). First, it was examined whether we could detect the same mitochondrial haplotype from the rearing water and of Ayu individual maintained in that corresponding tank. Second, it was examined whether we could correctly detect variation in mitochondrial haplotypes from an eDNA sample containing multiple haplotypes derived from

multiple individuals of Ayu. Multiple library replicates were prepared for the eDNA sample and sequenced them separately. The numbers of true haplotypes and false positive haplotypes were compared between the results obtained with and without the use of ASV methods for processing the HTS data. During the analysis, special emphasis was placed on the detection rate of each haplotype in library replicates because erroneous sequences are expected to occur randomly during experimental processes (e.g., PCR and MiSeq sequencing), and false positive haplotypes are expected to be detected rarely in multiple library replicates. Here it was expected that false positive haplotypes could be eliminated correctly from HTS data of eDNA sample by using ASV methods and/or removing haplotypes with low detection rates among library replicates.

2. Material and Methods

2.1. Primer design

The mitochondrial D-loop region was targeted, because it has a higher mutation rate compared with the nuclear DNA regions and the other mtDNA regions (Moritz et al. 1987). To amplify the control region of Ayu, two sets of species-specific primers were developed based on the complete mitochondrial DNA sequence of Ayu from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/, accession numbers of collected sequences were AB047553, EU12467–EU124683). The first primer set, Paa-Dlp-1 primer, was developed for Sanger-sequencing, which can amplify the nearly entire D-loop region (amplicon length, 541-bp). The sequences of the primers are as follows:

PaaDlp-1_F (5′-GCTCCGGTTGCATATATGGACC-3′),

PaaDlp-1_R (5′-AGGTCCAGTTCAACCTTCAGACA-3′)

The second primer set, PaaDlp-2, was designed for HTS by referring to instructions suggested previously (Miya et al. 2015; Palumbi 1996). Total 232 sequences of the mtDNA control region was obtained from Ayu from the MitoFish v.2.80 (Iwasaki et al. 2013; http://mitofish.aori.u-tokyo.ac.jp/)

and aligned these sequences. The information for all sequence data used to design primers for HTS (PaaDlp-2 primers) is listed in Table S4–1. The aligned sequences were imported into MESQUITE v. 2.75 (Maddison et al. 2010), and the search for a short hypervariable region (up to 200-bp for paired-end sequencing using the Illumina MiSeq) flanked by two conservative regions (ca 20–30 bp) was performed in the entire region of aligned sequences. For HTS, the PaaDlp-2 primers were designed on the selected positions within the amplification range of PaaDlp-1, considering the unconventional base pairing in the T/G bond to enhance the primer annealing (i.e. the designed primers use G rather than A when the template is variable C or T, and T rather than C when the template is A or G, Fig. 4–1). Two types of reverse primers, PaaDlp2_1R and PaaDlp2_2R, were designed, because the reverse priming sites has one variable site (the template is A or G) that does not bind despite the T/G bond. The base R indicates A (PaaDlp-2_R1) or G (PaaDlp-2_R2). The primer sequences are as follows:

PaaDlp-2_F (5′-CCGGTTGCATATATGGACCTATTAC-3′),

PaaDlp-2_R1 and PaaDlp-2_R2 (5′- GCTATTRTAGTCTGGTAACGCAAG -3′).

To check the specificity of the PaaDlp-1(F/R) and PaaDlp-2(F/R1/R2), an *in silico* specificity screen was performed using Primer-BLAST with default settings (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

*2.2.Haplotype determination from tank water eDNA and corresponding individual*A toalof 20 juveniles of Ayu (0.92 ± 0.21 g wet weight, mean ± SD) that were caught by a large fixed net in Lake Biwa (35°18'25" N; 136°3'40" E, DMS) in Japan on 24 February 2015 were purched from the fishermen. Live fish were brought back to the laboratory and then maintained individually in a small tank with 300 mL of aged tap water at room temperature. After 15 min, each fish was removed from the tanks and anaesthetized with an overdose of clove oil. To extract DNA from the tissues, about 0.02 g of skeletal muscle tissues was collected from each individual. DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used following the manufacturer’s protocol.

To collect eDNA, 250 mL of rearing water was sampled from each tank and vacuum-filtered using a Whatman GF/F glass fiber filter (GE Healthcare Japan, Tokyo, Japan; diameter 47 mm; nominal pore size of 0.7 μm). All filter disks were folded in half inward with tweezers and wrapped in aluminum foil, then stored at −20°C. eDNA was extracted according to the methods described in the section, 'eDNA extraction from filters'. As a filtration negative control (FNC), the same volume of ultrapure water was filtered in the same manner after the filtration of the all real samples. The FNC was treated alongside the samples in the following experimental steps to confirm no cross contamination. Before use, all sampling and filtration equipment were exposed to a 10% blech solution for 10 min, washed with running tap water and rinsed with ultrapure water. PCR was performed in a 25-μL reaction for each sample using the StepOnePlus Real-Time PCR System. The mixture of the reaction was as follows: 900 nM each of PaaDlp-1(F/R) in 1 × PCR master mix (TaqMan gene Expression Master Mix, Life Technologies, Carlsbad, CA, USA) with 7 μL of sample eDNA or 1 μL of tissue-derived DNA (10 ng/μL). The PCR thermal conditions were 2 min at 50ºC, 10 min at 95ºC, 44 cycles of 15 s at 95ºC, and 60 s at 60ºC. The PCR products were purified using Nucleo Spin® Gel and PCR Clean-up Kits (Code No. 740609.50; TAKARA Bio, Kusatsu, Japan) according to the manufacture's instructions. Sequences were determined by commercial Sanger sequencing service (Takara Bio, Kusatsu, Japan). The sequences which were successfully determined (total 448 bp) were deposited in the DNA database of Japan (DDBJ, https://www.ddbj.nig.ac.jp/dra/index.html; accession numbers, LC406364- LC406383) and are listed in Table S4–2.

2.3. eDNA extraction from filters

The filter samples were subjected to eDNA extraction following the method described in Yamanaka et al. (2017). The filter was rolled into a cylindrical shape using sterile forceps and placed in the upper part of a spin column (EZ-10; Bio Basic, Markham, Ontario, Canada) which was removed the silica membrane before use. Excess water remaining in the filters was removed by centrifugation for 1 min at 6000 *g*, and a mixture of 200 μL of ultrapure water, 100 μL of Buffer AL and 10 μL of proteinase K was dispensed onto the filter in each spin column and incubated for 15 min at 56ºC. The Buffer AL

and proteinase K were supplied from the DNeasy Blood & Tissue Kit. After incubation, the spin columns were centrifuged for 1 min at 6000 *g* to elute the eDNA into 2-mL tube. The upper part of the spin column was placed in a new 2-mL tube, and 200 µL of Tris-EDTA buffer (pH 8.0) was added to the filter and incubated for 1 min at room temperature to recover the remaining DNA on the filter. The spin columns were centrifuged for 1 min at 6000 *g* to obtain the second elution and mixed with the first elution. Subsequently, 100 µL Buffer AL and 600 µL ethanol were added to each tube and mixed by pipetting. The eDNA then was collected and purified from each solution using the DNeasy Blood & Tissue Kit following the manufacturer's protocol, with the minor modification that the final elution volume was adjusted to 100 µL of Buffer AE.

2.4. Detection of mitochondrial haplotype diversity from an eDNA sample

The experimental design is shown in Fig. 4–2. 50 mL of rearing water was collected from each tank used in the experiment described above. All collected water was mixed (total volume 1 L) and vacuum-filtered using a Whatman GF/F glass fiber filter. eDNA was extracted according to the methods described in above. After extracting eDNA from the filter sample, a two-step tailed PCR approach was employed to construct paired-end sequencing libraries, according to methods described by Miya et al. (2015). The FNC sample in the first experiment was used again as FNC sample in this experiment, because the filtrations of both experiments were performed at the same time. To avoid the risk of cross-contamination, all sampling and filtering equipment were decontaminated with 10% bleach solution for more than 10 min, carefully washed with tap water, and finally rinsed with ultrapure water. In addition, the PCR set-up was performed in a different room from PCR and HTS. The first PCR was performed in five replicates, each in a 12-µL reaction for a sample. The target region of Ayu was amplified using primers containing adapter sequences and random hexamers (N). The primer sequences are as follows:

5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNN + PaaDlp-2_F (gene-specific sequences) -3′ and 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNN +

PaaDlp-2_R1/R2 (gene-specific sequences) -3′. The mixture of the reaction was as follows: 0.3 μM of PaaDlp-2_F, 0.15 μM each of PaaDlp-2_R1 and R2 in 1 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, WA, USA) and a 2-μL sample of eDNA. To monitor cross contamination during library preparation, non-template control (NTC) were included in triplicate in the first PCR. The PCR thermal conditions were 3 min at 95ºC, 35 cycles of 20 s at 98ºC 15 s at 60ºC, and 15 s at 72ºC, followed by a final extension for 5 min at 72ºC. The first PCR products were purified twice using Agencourt AMPure XP beads (Beckman Coulter), according to the manufacturer's instructions (reaction ratio; AMPure beads 0.8: PCR product 1, target amplicon length; ca. 290 bp).

The second PCR was performed in three replicates, with each a 12-μL reaction of the first PCR (total 15 replicates per sample). To distinguish library replicates during Illumina MiSeq sequencing, respective library replicates (total 15 replicates) were indexed with different combinations of indexing primers. The primer sequences used in second PCR are listed in Table S4–3. The mixture of the reaction was as follows: 0.3 μM of each second PCR primer in 1 × KAPA HiFi HotStart ReadyMix and 2 μL of the purified first PCR product from the Agencourt AMPure XP beads. As negative controls in the second PCR, 2 μL of the first PCR product of the NTC was added to each reaction instead of template eDNA. The PCR thermal conditions were 3 min at 95ºC, 12 cycles of 20 s at 98ºC and 15 s at 65ºC, with a final extension for 5 min at 72ºC. The indexed second PCR products were pooled in equal volumes (5 μL each). The target size of the libraries (ca. 370 bp) was collected using 2% E-Gel® SizeSelect™ agarose gels (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

DNA concentrations in the collected libraries were estimated using the Qubit fluorometer (Life Technologies) with the Qubit dsDNA HS assay kit and adjusted to 4 nM (assuming 1 bp equals 660 g mol^{-1}) using ultrapure water. A 5-μL of the 4 nM library was denatured with 5 μL of fresh 0.2 N NaOH, followed by 5 μL of Tris HCl (200 mM, pH 7) and 985 μL of HT1 buffer (including in Miseq Regent Kit) was added to adjust the library concentration to 20 pM. Then, 48 μL of 20 pM PhiX DNA (Illumina, San Diego) and 360 μL of HT1 buffer were added to 192 μL of the 20 pM library to obtain

a 8 pM library. The library was sequenced using the MiSeq platform (Illumina, San Diego), with the MiSeq v2 Regent Kit for the 2 × 150 bp PE cartridge (Illumina, San Diego). The sequencing reads obtained in the present study were deposited in the DDBJ Sequence Read Archive (accession number: DRA006638).

The MiSeq paired-end sequencing (2 × 150 bp) of the 21 libraries for this study (including 15 library replicates, three FNC and three NTC), together with 105 libraries from the other study (total number of libraries =126), yielded a total of 15.98 million reads, with 97.5% base calls containing Phred quality scores greater than or equal to 30.0 (Q30; error rate = 0.1% or base call accuracy = 99.9%).

2.5. Bioinformatic analysis

The full range of amplicons obtained using the PaaDlp-2 primers were successfully sequenced using the MiSeq platform. However, for the amplicons obtained using the PaaDlp-1 primers, some bases following the forward primer were undetermined by Sanger sequencing of the tissue-derived DNA from 20 individuals of Ayu. The forward primers of PaaDlp-2 and PaaDlp-1 were designed to be close to each other, and thus, three bases after the forward primer of PaaDlp-2 were needed to be omitted to compare the overlapping regions between the two datasets. Therefore, only 163 of the bases successfully determined for the two datasets were used for the subsequent bioinformatic analyses.

To perform a correction for erroneous sequences based on the ASV method, fastq files containing raw reads were processed using the Divisive Amplicon Denoising Algorithm 2 package ver. 1. 6. 0 (DADA2, Callahan et al. 2016) of R. The core algorithm of DADA2 infers unique biological variants using the denoising algorithm that is based on a model of errors in the amplicon sequencing with MiSeq. The detailed algorithm of DADA2 is described in the original paper. Briefly, the adopted error model in DADA2 quantifies the rate λji, at which an amplicon read with sequence i is produced from sample sequence j as a function of sequence composition and quality. Then, the p-value of the null hypothesis that the number of amplicon reads of sequence i is consistent with that of the error model

was calculated using a Poisson model for the number of repeated observations of the sequence *i*, parameterized by the rate λji. Calculated *p*-values were used as a division criterion for an iterative partitioning algorithm, and sequence reads were divided until all partitions were consistent with being produced from their central sequence. Reads of sequences inferred as error were replaced with the central sequence of the partition that included its sequence (i.e. error correction).

In this study, reads with one or more expected errors (maxEE = 1) were discarded during quality inspection and trimming of primer sequences. Quality-filtered sequences were dereplicated, and the parameters of the DADA2 error model were trained on a random subset of one million reads. The trained error model was used to identify and correct indel-mutations and substitutions. Denoised forward and reverse reads were merged and read pairs with one or more conflicting bases between the forward and reverse read were removed. DADA2 implements the function 'removeBimeraDenovo' to identify chimeras; however, it was not used in this study because haplotypes of Ayu included in sample water might be incorrectly identified as chimeras due to high sequence similarity. All detected sequences were confirmed to be 100% identical to the Ayu sequences determined in previous studies, using nucleotide BLAST (basic local alignment sequence tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Furthermore, the HTS data also was processed without the ASV method. The base calling errors were eliminated by quality filtering. The data pre-processing and dereplicating were performed using a custom pipeline described by Sato et al. (2018). Briefly, the low-quality tails were trimmed from each read, and the tail-trimmed paired-end reads (reads 1 and 2) were assembled using the software FLASH with a minimum overlap of 10-bp. The primer sequences were then removed with a maximum of three-base mismatches. Only when sequences had 100% identity with each other, they were operationally considered as identical. The fastq format was transformed into fasta, and the pre-processed reads were dereplicated. At this point, the reads were subjected to a local BLASTN search against a custom-made database of the control region of Ayu. The custom-made database was

constructed from the 190 haplotypes of mitochondrial D-loop region of Ayu, which represented the sequences downloaded from MitoFish but excluded the 96 individuals (accession number LC406384-LC406403, Table S4–4) caught at Ado river (35°19'31" N, 136°3'48" E, DMS, Japan). The Ado river is connected to Lake Biwa and located close to a large fixed net (ca. 4 km) that was used to catch Ayu juveniles for the present study. The information in all haplotypes included in the custom-made database is listed in Table S4–4. If the respective sequences obtained in the HTS data had ≥ 99% similarity with the reference haplotype and an E-value < 10^{-5} in the BLAST results, the sequences were identified as those of Ayu.

2.6. Statistical analysis

All statistical analyses were performed using R ver. 3. 2. 3 software (R Core Team. 2016) and the minimum level of significance was set at $\alpha = 0.05$. To determine differences in the total number of reads derived from the nine true haplotypes, which were derived from 20 individuals (see "Results"), and false positive haplotypes, a Mann–Whitney *U* test was performed. A generalized linear model (GLM) with Poisson distribution was used to test how the detection rate of false haplotypes in the library replicates affected the total reads of the false haplotypes (glm function in R ver. 3.2.3 software). To visually determine the genetic distances among haplotypes that were detected from 15/15 library replicates (see "Results") and the relative read abundance of these haplotypes, haplotype network was generated using ape v5.1 and pegas v0.11 packages of R (Paradis et al. 2004).

3. Results

3.1. Testing species specificity of the two primer sets

The *in silico* specificity check for PaaDlp-1 and PaaDlp-2 (no adapter sequence) implemented in Primer-BLAST indicated species-specific amplification of Ayu. The direct sequencing of the PCR amplicons corroborated the amplification of the target region of Ayu in section 'Haplotype determination from tank water eDNA and corresponding individual'.

3.2. Comparison of detected haplotypes from tissue-derived DNA and corresponding tank eDNA

The sequences from the 20 Ayu individuals that were used for tank experiments were classified into 17 and nine haplotypes based on Sanger sequencing of PCR products amplified using PaaDlp-1 (amplicon length: 448 bp) and PaaDlp-2 (amplicon length: 163 bp), respectively (Table S4–2 and Fig. S4–1). The detected haplotypes had only one or a few differences from each other, with the maximum pairwise *p*-distances for the two datasets being 0.022 (PaaDlp-1) and 0.025 (PaaDlp-2), respectively. Each sequence obtained from eDNA, which was amplified using PaaDlp-1 and was detected from each of the 20 rearing water tanks, was identical to that obtained from tissue-derived DNA of the corresponding individual. In this tank experiment, the target fragments were not detected in any FNC and NTC. Thus, there was no evidence for cross-contamination during sample processing.

3.3. Detection of mitochondrial haplotype diversity from an eDNA sample

Based on the bioinformatic analysis using the ASV method, 1,539,351 reads were detected and assigned to 39 haplotypes (Fig. 4–3a, Table S4–5). Of these, 1,471,385 (96%) reads were assigned to eight true haplotypes and they were detected from 15/15 of library replicates. The remaining 67,966 (4%) reads consisted of 31 false positive haplotypes. A total of 386 (0.025%) and 235 (0.015%) reads were detected from the three FNC and three NTC (Table S4–5), respectively. The 31 false positive haplotypes were detected from 1/15 to 15/15 detection rates, but seven false positive haplotypes were detected all 15 library replicates. In addition, the false positive haplotypes with a low detection rate were randomly detected from the 15 library replicates on each filter and were not derived from any particular first PCR replication (Table S4–5).

Based on the bioinformatic analysis without the ASV method, 1,748,030 reads out of the total reads that passed quality control processes were assigned to Ayu with greater than or equal to 99% identity to the reference haplotypes in the custom-made database. Of these, 1,502,828 (86%) reads were assigned to nine true haplotypes, and they were detected from 15/15 library replicates. The remaining 245,202 (14%) reads consisted of 5,683 false positive haplotypes. The 5,683 false positive haplotypes were detected in 1/15 to 15/15 detection rates; however, 335 false positive haplotypes were detected in 15/15 library replicates (Fig. 4–3b). Despite the efforts to avoid the risk of cross-contamination, 124 (0.007%) and 105 (0.006%) reads were detected from the three FNC and three NTC, respectively.

Regardless of whether the ASV method was used, read abundances of the true haplotypes were significantly larger than those of the false positive haplotypes (Mann-Whitney *U* test; with ASV method, $p < 0.001$, $z = 4.27$; without ASV method, $p < 0.001$, $z = 5.21$; Fig. 4–3a and b). Furthermore, the total reads of false positive haplotypes increased significantly by increasing the detection rate in library replicates (GLM; $p < 0.001$, $p < 0.001$; Fig. 4–3a and b).

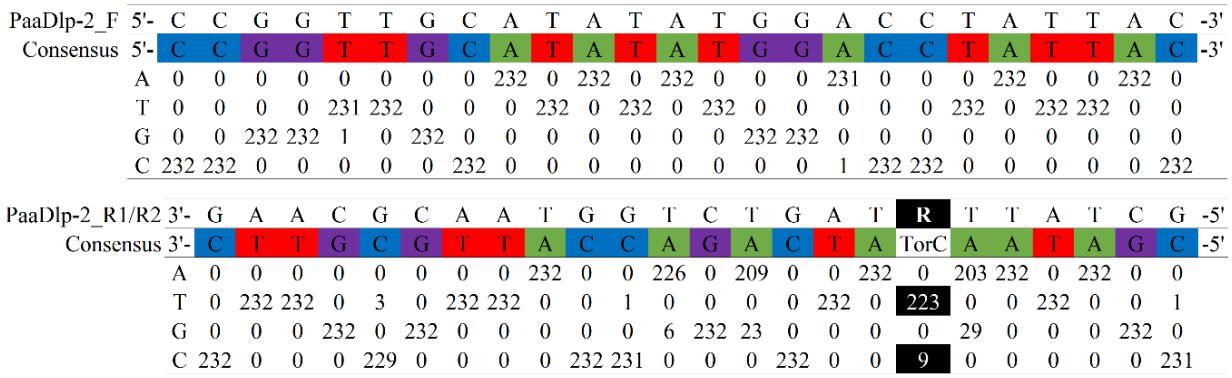

Fig. 4–1. The sequences of the PaaDlp-2 primers and sequence variation in the corresponding region. (Downloaded data from MitoFish v.2.80.) The background black indicates that the base does not bind despite the T/G bond. Note the presence of nucleotide substitutions only in one sequence out of 232, which was ignored during primer design.

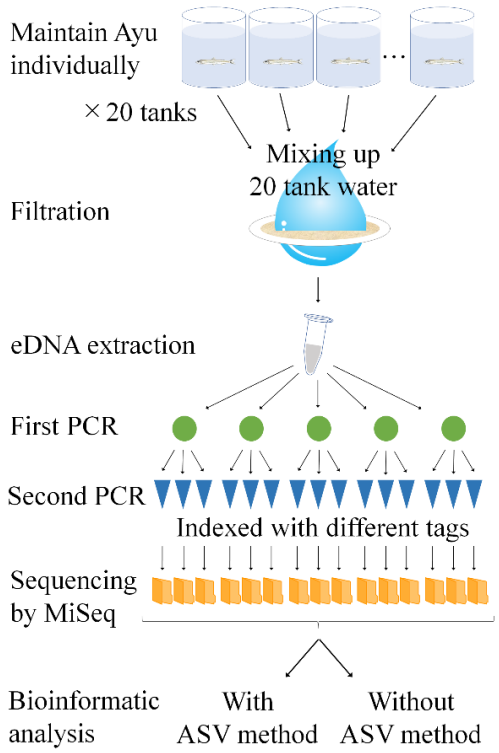

Fig. 4–2. Experimental design for detecting mitochondrial haplotype diversity from an eDNA sample.

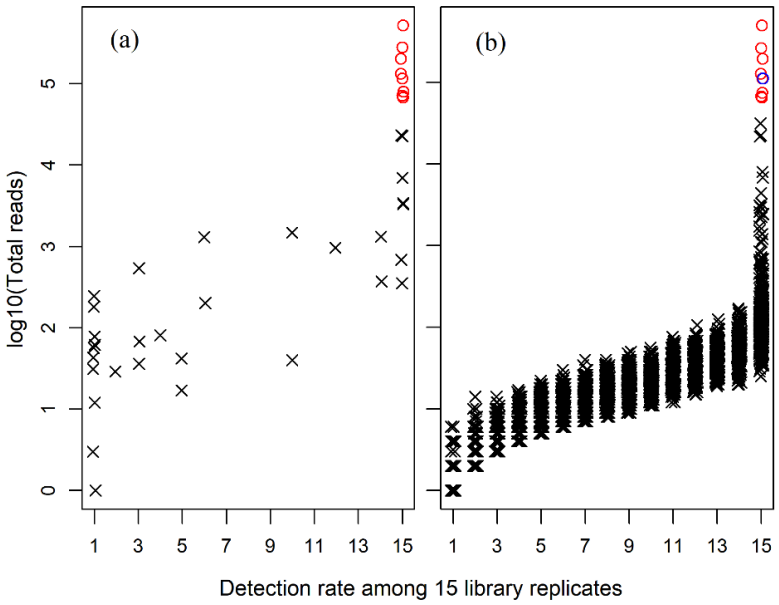

Fig. 4–3. Relationships between detection rate and total reads for each haplotype. They were detected (a) with and (b) without the ASV method. The red circle and cross indicate the true haplotype and false positive haplotype, respectively. The blue circle indicates the true haplotypes that were detected without using the ASV method but were not detected with the ASV method (false negative haplotype; True haplotype ID I).

4. Discussion

It was found that correcting erroneous sequences with the ASV method was effective to improve the accuracy of intraspecific diversity evaluates with eDNA analysis. Furthermore, the accuracy of the analysis seems to be further improved by removing of haplotypes with low detection rates. Although some caution is still required for risk of false positives and false negatives, the proposed approach is useful for applying eDNA analysis to evaluation of intraspecific diversity that requires higher accuracy with respect to distinguishing true haplotypes from false ones.

The use of the ASV method in eDNA analysis for evaluating intraspecific diversity considerably decreased the number of false positive haplotypes (Fig. 4–3a and b). The great performance of the ASV method for eliminating false positive haplotypes is consistent with previous studies that identified microorganisms from mock community samples at fine taxonomical resolutions (Callahan et al. 2016, Hughes et al. 2017, Kopylova et al. 2016). The ASV method can correct a large proportion of erroneous sequences in HTS data, and thus, the combination of the ASV method and eDNA analysis has great potential to advance studies of intraspecific diversity of aquatic macroorganisms. In this study, the DADA2 package was used as an ASV method, but some other Illumina denoisers, which are based on different algorithms also have been published, including UNOISE (Edgar and Flyvbjerg 2014), MED (Eren et al. 2015) and UNOISE2 (Edgar et al. 2016). The number and variety of false positive and false negative are likely to change depending on the method used, and the detection accuracy is expected to be improved by future development of ASV methods. Thus, novel ASV methods must be evaluated critically in future studies to increase the accuracy intraspecific diversity evaluates based on eDNA analysis.

There was one true haplotype (ID I), which was a false negative when the ASV method was used for bioinformatic analysis (Fig. 4–3a and b; Table S4–5). If there was variation within the priming site of the PaaDlp-2 primers, it could cause failure in PCR amplification (cf. Miya et al. 2015). However, haplotype ID I was a perfect match with the primer sequence of PaaDlp-2. In addition, when the HTS data was analyzed without the ASV method, it was detected from all 15 library replicates with a higher number of reads than false positive haplotypes. These results suggest that the DNA fragment of haplotype ID I was successfully amplified in PCR for library preparation. In other words, the sequence of true haplotype ID I was identified incorrectly as an error sequence and was judged as a false haplotype by ASV method even though the haplotype reads were present in all 15 library replicates. This false negative result is known to be caused by a failure to infer unique biological variants in the DADA algorithm (Rosen et al. 2012). In the DADA2 algorithm, haplotypes at low abundance (sequence reads) that have sequences similar to highly abundant (and true) haplotypes are more likely to be corrected and merged with the similar (true) haplotypes (cf. Callahan et al. 2016). The true haplotype ID I had one or two base pair differences with the other true haplotypes (ID B, E and F) and was mapped in close proximity to the abundant true haplotypes (Fig 4–4). In addition, the eight true haplotypes were detected with read abundances higher than the false positive haplotypes. Considering these results, the sequences of true haplotype ID I might have been identified as an error haplotype and corrected by DADA2.

The present results suggest that detection rates of each haplotype in library replicates provide an important clue to discriminate true haplotypes from false positive haplotypes. True haplotypes, especially predominant haplotypes, would be amplified at an early stage of PCR in all library replicates. However, unlike true haplotype, false positive haplotypes would not be contained in the initial eDNA template, but they are stochastically generated in some library replicates at a low rate (Fukui et al. 2013, Nakamura et al. 2011). The false positive haplotypes with low detection rates in this study were incidentally generated in the first and second PCR step with no detection pattern.

Furthermore, detection rates of true haplotypes in library replicates are expected to be much higher than those of false positive haplotypes. This expectation was strongly supported in this study (Fig. 4–3a and b). It was found that the false positive haplotypes were successfully eliminated by combination of the use of the ASV method and the remove sequences that had detection rates less than 15/15 in library replicates. Therefore, the accuracy of evaluation of intraspecific diversity using eDNA analysis would be increased further by selecting haplotypes with high detection rates among multiple library replicates.

The present study also has implications for understanding the relationship between the total reads of each haplotype and the number of individuals owning that haplotype. Previous studies have suggested that the eDNA concentration increases with an increase in abundance and/or biomass of organisms (Doi et al. 2016, Pilliod et al. 2013, Takahara et al. 2012, Yamamoto et al. 2016). In addition, eDNA sequence reads were likely correlated with biomass and the number of individuals belonging to each fish family in the community (Thomsen et al. 2016). Thus, there is a possibility that the total number of reads of each true haplotype reflects the number of individuals that represent corresponding haplotypes (hereafter called 'owner individuals'). In this study, using the GLM analysis with the Poisson distribution, the number of owner individuals had a significant positive effect on the total reads of the haplotypes regardless of whether the ASV method was used ($p < 0.01$ in both cases; Fig. 4–5). This result suggests that the use of eDNA analysis has the potential to evaluate not only the diversity of haplotypes but also the relative dominance of each haplotype in a population. Furthermore, the total read abundance potentially can be used to eliminate false positive haplotypes based on an appropriate threshold value. However, this approach was consciously avoided. In a field setting, *a priori* information on the haplotype composition in the target population and the relative concentration of eDNA corresponding to each haplotype are usually unknown, and thus, the use of a higher threshold value for the total read abundance may lead to eliminate true haplotype and increase false negative rates.

In future studies, it will be necessary to address whether we can accurately detect true haplotypes derived from wild populations, because eDNA concentrations of field samples will be lower than those in tank experiments (e.g. Minamoto et al. 2017, Takahara et al. 2012). In addition, heterogeneous distributions of eDNA in field water has been also reported (Dejean et al. 2012, Jerde et al. 2011, Pilliod et al. 2013, Thomsen et al. 2012, Yamamoto et al. 2016). Therefore, it is necessary to determine optimal sampling strategies, including appropriate volumes of sample water and distances between sampling points to accurately evaluate intraspecific diversity using eDNA analysis. Further development of eDNA analysis to evaluate intraspecific diversity would contribute to more effective genetic resource management and ecosystem monitoring.

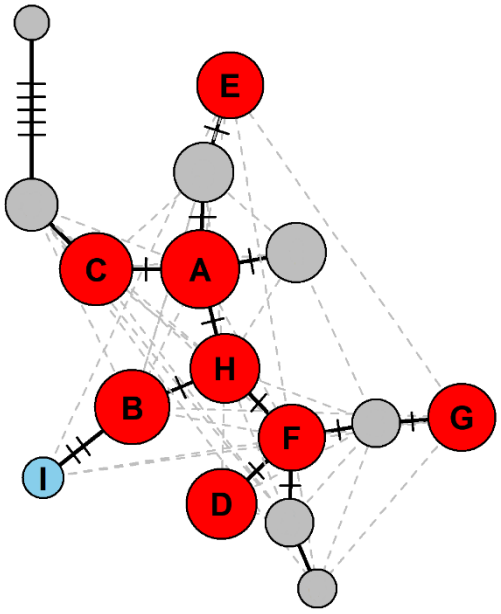

Fig. 4–4. Haplotype network of the true haplotypes and the false negative haplotypes detected from 15/15 library replications. Red, grey and blue circles indicate true haplotypes, false positive haplotypes and false negative haplotypes, respectively. The alphabet indicates each haplotype ID, and the circle size reflect the read abundance of each haplotype. Each of the tick marks corresponds to one base pair difference. Dashed lines among haplotypes represent one or two base pair difference.

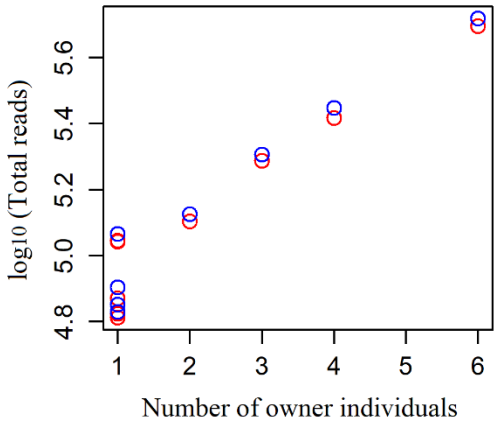

Fig. 4–5. Relationship between the number of owner individuals and total reads for each detected true haplotype. Blue and red circles indicate true haplotypes detected with and without the ASV method, respectively. The number of owner individuals of each haplotype had a significant positive effect on the total reads with and without using the ASV method ($p < 0.01$, $p < 0.01$, respectively).

5. Data Archiving Statement

The minimal raw dataset is uploaded to the DDBJ Sequence Read Archive https://www.ddbj.nig.ac.jp/dra/index-e.html; Accession number: DRA006638).

6. Supplementary information

Table S4−1. The information of all sequence data which were used for designing the PaaDlp-2 primers.

Table S4–2. Detected sequence haplotypes from 20 individuals of ayu.

Table S4–3. Primer sequences for second PCR.

Table S4–4. The information of all haplotypes included in custom-made database. Sequences of accession number LC406384- LC406403 were newly deposited sequences.

Table S4–5. The All haplotypes which were detected by ASV method and its reads on each library. Haplotype ID2 correspond to table S4-2, dash mark (-) indicate the false positive haplotype.

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Chapter V

Potential of the environmental DNA analysis for estimating the intraspecific diversity in a wild fish population

1. Introduction

Intraspecific genetic diversity is an important component of biodiversity (Allendorf et al. 2012, Laikre et al. 2016) and it affects ecological and evolutionary processes (Wolf and Weissing 2012). However, estimation of the intraspecific diversity using conventional methods is generally laborious and invasive because target organisms need to be captured for tissue sampling. Moreover, especially when a rare and endangered species are targeted, conventional methods may threaten the persistence of species or population because they potentially damage individuals and their habitats. Also, in laboratories, the cost in time and labor would substantially increase depending on the number of samples and study sites because one-by-one Sanger sequencing of each sample is required. Therefore, the development of efficient and non-invasive method would promote estimating the intraspecific diversity in ecosystem management.

Environmental DNA (eDNA) analysis has been used as a new useful approach for investigating the species distribution of aquatic macroorganisms (e.g. Ficetola et al. 2008, Jerde et al. 2011, Takahara et al. 2013, Rees et al. 2015). Environmental DNA analysis enables us to detect the presence of species efficiently and non-invasively by analyzing eDNA contained in water samples instead of capturing or observing the target species (Rees et al. 2014, Fukumoto et al. 2015, Thomsen et al. 2015, Yamanaka and Minamoto 2016). More recently, combined with high-throughput sequencing (HTS) technology, eDNA metabarcoding provides information on species diversity by determining multiple sequences in eDNA samples (e.g. Miya et al. 2015, Shaw et al. 2016, Thomsen et al. 2016, Ushio et al. 2017, Yamamoto et al. 2017). Furthermore, Sigsgaard et al. (2016) first reported the

applicability of eDNA analysis for estimation of their intraspecific diversity. They detected multiple mitochondrial haplotypes derived from the whale shark (*Rhincodon typus*) wild populations in eDNA sample by using HTS.

However, the HTS data always contain erroneous sequences which are derived from DNA polymerase errors during PCR and sequencing errors (cf. Schloss et al. 2011, Coissac et al. 2012, Edgar et al. 2016). It is difficult to distinguish erroneous sequences from the original sequences, because the most of erroneous sequences are very similar to original sequences and are masked by intraspecific diversity. Accordingly, results of eDNA analysis using HTS technology for estimating the intraspecific diversity contain false positive (FP) haplotypes and cause overestimates of diversity.

To address this problem, it was previously proposed that tha use of a set of techniques, namely amplicon sequence variant (ASV) method and haplotype selection based on detection rate, to minimize the number of false positive haplotypes (Tsuji et al. 2018). ASV methods are new bioinformatic methods that denoise HTS data based on error models, and it has been used in the field of microbial ecology (e.g. Mora et al. 2016, Boeck et al. 2017, Bond et al. 2017). ASV methods allows us to distinguish erroneous sequences that has one or few base-pair substitutions, because it does not require operational taxonomic unit clustering of similar sequences (Callahan et al. 2017). On the other hand, the haplotype selection was performed based on the assumption that false positive haplotypes have lower detection rate than original haplotypes among multiple library replications because error sequences are randomly generated during PCR or HTS. The proposed techniques successfully eliminated 99.5% of false positive haplotypes in the previous study that used aquarium water of Ayu fish (*Plecoglossus altivelis altivelis*) (Tsuji et al. 2018). However, it is not clear yet that the applicability of the same techniques to the field, because field water tends to contain a variety of haplotypes at lower concentration than aquarium water. Therefore, it is

necessary to examine whether we could correctly detect present haplotype in field water using our proposed techniques.

The objective of this study was to examine whether eDNA analysis with the proposed ASV method and haplotype selection could correctly estimate intraspecific diversity of a wild fish population. The water sampling and the capturing of Ayu specimens were performed at the same location in a river on the same day. It was investigated how many haplotypes obtained by a conventional method were detected by eDNA analysis with the proposed techniques for denoising HTS data. In addition, water sample was divided onto multiple filters and library replications to examine possible miss sampling and amplification of scarce haplotypes. Based on the accumulation curve of detected haplotype number against replication numbers, it was estimated that the required number of filter and library replications to reach a steady state in the number of detected present haplotypes.

2. Materials and methods

2.1. Sampling sites and experimental design

A water sample and fish specimens were collected in the lower reach of Ado River (35°19'30" N, 136°03'49" E), a tributary of Lake Biwa, Japan, on May 1, 2015, when numerous individuals of Ayu were migrating upstream from Lake Biwa. To avoid contamination, different investigators performed water sampling and purchasing of Ayu. The entire experimental design was showed in Fig. 5–1.

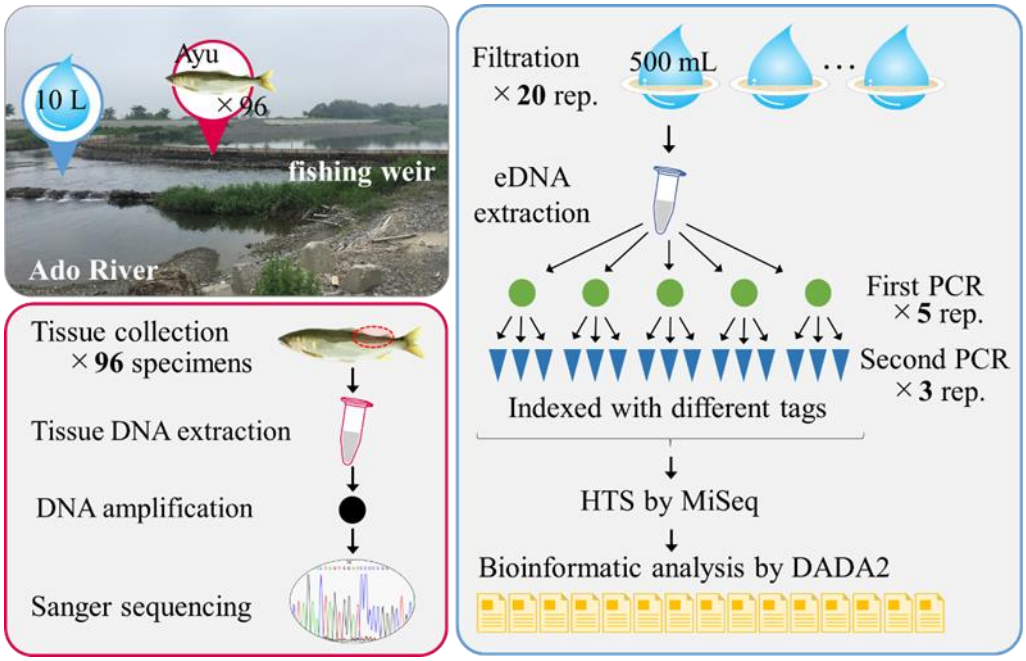

Fig. 5–1 Experimental design.

2.2. Determination of sequence from captured specimens by Sanger sequencing

A total of 96 specimens of Ayu (11.43 ± 1.84 g wet weight, mean ± SD) were purchased from the fishermen. They were captured within 12 hours before water sampling by a fishing weir located approximately 30 m upper from the water sampling point. Captured fish were quickly preserved using ice. In a laboratory, approximately 0.02 g of the skeletal muscle tissue was collected from each specimen, and tissue DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The tissue DNA was finally eluted in 200 µL of Buffer AE. For Sanger sequencing, the nearly entire control region of mitochondrial DNA was amplified using PaaDlp-1_F (5'-GCTCCGGTTGCATATATGGACC-3') and PaaDlp-1_R (5'-AGGTCCAGTTCAACCTTCAGACA-3') (Tsuji et al. 2018) by StepOnePlus Real-Time PCR System (Life Technologies, CA, USA). 20-µL reaction mixture contained 900 nM of PaaDlp-1(F/R) in 1 × PCR master mix (TaqMan gene Expression Master Mix), and 1 µL of tissue-derived DNA (10 ng/µL). The thermal cycle profile was 2 min at 50 ºC, 10 min at 95 ºC, 44 cycles of 15 s at 95 ºC, and 60 s at 60 ºC. Sequences of the PCR amplicons were determined by commercial

Sanger-sequencing service (Takara Bio Inc. Kusatsu, Japan). The sequences were deposited to the DNA database of Japan, and the accession numbers are LC433925–LC433966.

2.3. Water sampling and filtration for eDNA analysis

Ten litters of surface water was collected using a plastic tank. The water quality parameters in separately sampled water measured using water quality sensors (HI 98130; HANNA Instruments, Woonsocket, RI, USA) were as follows: pH 6.35, temperature 17.4°C, and electrical conductivity 0.05 mS/cm. Immediately after agitation of the whole 10 L water sample, 500 mL of water sample was filtered on site onto each of 20 glass fiber filters (Whatman GF/F, 0.7-µm mesh, GE Healthcare, Chicago, USA). As a filtration negative control, the 500 mL of ultrapure water was filtered on site and treated in the same manner in the following experiments as the real samples. The filter discs were immediately stored at −20°C until eDNA extraction. To avoid contamination, all sampling and filtering equipment were soaked in 10% bleach solution for more than 10 min, carefully washed with tap water, and finally rinsed with ultrapure water before use.

2.4. eDNA extraction from filter samples

Environmental DNA was extracted from the filter samples following the procedures of Yamanaka et al. (2017). Each filter disc was placed into a spin column (EZ-10 SpinColumn & Collection Tube; Bio Basic Inc., Ontario, Canada), from which a silica-gel membrane was prospectively removed, and excess water on the filter was removed by centrifugation. Then, the mixture, containing 200-µL ultrapure water, 100-µL buffer AL, and 10-µL proteinase K, was added onto the filter and incubated at 56 ºC for 15 min. After centrifuged at 6000 × *g* for 1 min, upper parts of the spin columns were removed and placed on new 2-mL collection tubes. Then, 200 µL of TE buffer (pH 8.0) was added onto each filter to recover residual DNA on the filter and incubated again at room temperature for 1 min. After centrifuged at 6000 × *g* for 1 min, the elution was mixed with their first filtrates, 200 µL of buffer AL and 600 µL of 100% ethanol. The mixture was then applied to a DNeasy Mini Spin

Column, which were supplied by DNeasy Blood & Tissue Kit, and centrifuged at 6000 × *g* for 1 min. According to the manufacturer's instructions, the DNA was purified and finally extracted with 100 μL of Buffer AE. The extracted DNA samples were stored at −20°C. The reagents, buffer AL, buffer AE and proteinase K, which were used for DNA extraction, were attachment reagents from DNeasy Blood & Tissue Kit.

2.5. Library preparation and paired-end sequencing by MiSeq

Paired-end library preparation and MiSeq sequencing were performed using same method which were described by Tsuji et al. (2018). The two-step tailed PCR approach was employed to construct the paired-end libraries. The first PCR was performed using a primer pairs, which can species-specifically amplify the control region of Ayu mitochondrial DNA. The primer pairs were developed in Tsuji et al. (2018) and contained adapter sequences and random hexamers (N). The sequence of primers are as follows: PaaDlp-2_F (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNCCGGTTGCATATATGGACC TATTAC-3'), PaaDlp-2_R1 and R2 (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNGCTATTRTAGTCTGGTAACG CAAG -3'). The R indicate A (PaaDlp-2_R1) or G (PaaDlp-2_R2). The PCR mix with total volume of 12 μL contained: 0.3 μM of PaaDlp-2_F, 0.15 μM of PaaDlp-2_R1 and R2 in 1 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), and 3 μL sample eDNA. The first PCR was performed with five replicates for each eDNA sample derived from 20 filter replications. In addition, triplicated non-template controls were included for each PCR run to monitor cross-contamination during the library preparation. The thermal cycle profile was 3 min at 95 ºC, 35 cycles of 20 s at 98 ºC, 15 s at 60 ºC, and 15 s at 72 ºC followed by the final extension for 5min at 72 ºC. The first PCR products were purified using the Agencourt AMPure XP (Beckman Coulter, USA) according to the manufacturer's instructions (reaction rate: AMPure beads 0.8: PCR product 1, target amplicon length: ca. 290-bp).

The second PCR was performed in triplicate for each replication of first PCR product (total 15 replications for each filter sample). The PCR mix with total volume of 12 μL contained: 0.3 μM of each second PCR primer in 1 × KAPA HiFi HotStart ReadyMix, and 3 μL of purified first PCR product. Samples were distinguished each other based on different combinations of indexing primers in bioinformatic analysis. The primer sequences used in second PCR are listed in Table S5–1. In non-template controls of the second PCR, the non-template controls in first PCR was added as template. The thermal cycle profile was 3 min at 95 ºC, 12 cycles of 20 s at 98 ºC and 15 s at 65 ºC, with the final extension for 5 min at 72 ºC.

All indexed second PCR products were pooled in equal volumes (1 μL each), and the target size of the libraries (ca. 370-bp) was collected using 2% E-Gel® SizeSelect™ Agarose Gels (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. DNA concentrations of the collected libraries were adjusted to 4 nM (assuming 1 bp of DNA has the molecular weight of 660 g) using ultrapure water. Finally, the libraries were sequenced on a single MiSeq run using an Illumina MiSeq v2 Reagent kit for 2 × 150 bp PE (Illumina, San Diego, CA, USA).

2.6. Bioinformatic analysis using ASV method

The full range of amplicon obtained using PaaDlp-2 primers were successfully sequenced by MiSeq platform; however, some bases after the forward primer of PaaDlp-1 primers were undetermined by the Sanger-sequencing of the tissue-derived DNA. Because the forward primers of PaaDlp-2 and PaaDlp-1 had designed at close sites, three bases after the forward primer of PaaDlp-2 were needed to be omitted to compare the overlapping regions between two datasets. Thus, only 163 bases which were successfully determined for both of two datasets were used for the subsequent bioinformatic analyses.

To perform the correction of erroneous sequences based on the ASV method, the fastq files including raw reads obtained from 15 library replicates derived from each filter sample were processed using the Divisive Amplicon Denoising Algorithm 2 package ver. 1. 6. 0 (DADA2,

Callahan et al. 2016) of R ver. 3. 2. 3 software (R Core Team 2016). The DADA2 is one of the ASV methods for denoising sequencing errors in Illumina-sequenced amplicons, and it has been used in the field of microbial ecology (Mora et al. 2016, Hughes et al. 2017, Schwendner et al. 2017). The denoising algorithm of DADA2 is based on pairwise comparison of sequences and uses quality scores of the reads as well as the probability of various copy errors that could be introduced during PCR amplification and sequencing.

First, the error model of DADA2 quantifies the rate λji, at which an amplicon read with sequence i, is produced from sample sequence j as a function of sequence composition and quality. Second, the p-value of the null hypothesis that the number of reads of sequence i is consistent with that of the error model was calculated using a Poisson model for the number of repeated observations of the sequence i, which is parameterized by the rate λji. Then, calculated p-values are used as division criteria for an iterative partitioning algorithm, and the reads of sequence continue to be divided until all partitions are consistent with being produced from their central sequence. Finally, to correct erroneous sequences, reads of the sequences inferred as error are replaced with the central sequence of the partition including the erroneous sequences. See Callahan et al. (2016) for full description of the denoising algorithm in DADA2.

In this study, reads were filtered and sequenced of random hexamer and primers were trimmed using the filterAndTrim function (parameters were as follows: maxN=0, truncQ=2, and maxEE=1). Passed sequences were dereplicated, and error rate was estimated using the DADA2 function and was used for error model. Reads of forward and reverse sequences were denoised using error model, and read pairs were merged using the mergePairs fanction. To identify chimeras, the DADA2 implements the function of “removeBimeraDenovo”. However, remove of chimeric sequences were not performed in this study because haplotypes of Ayu included in sample water might be incorrectly identified as chimeras due to the high sequence similarity.

2.7. Data analysis

In eDNA analysis, the haplotype detected from one or more library replications out of 15 were defined as detected haplotypes from each filter. For each haplotype, detection rate out of 15 library replications from each filter was calculated. To explore optimal number of filter replications for detecting present haplotypes, the accumulation curve was estimated using the function "specaccum" in the vegan v.2.5-1 package (Oksanen et al. 2018) of R based on the relationship between the number of detected present haplotypes and the number of analyzed filter. The accumulation curve was estimated using two data sets, namely 1) 15 library replications per filter (five first PCR replications × three second PCR replications.) and 2) five library replications per filter (five first PCR replications and only first replication out of three second PCR replications.), to examine the effect of the number of library replications per filter on the number of detected present haplotype. Generalized linear models (GLMs) with logit link function assuming binomial distribution were used to analyze the relationships between the number of filter or library replicates that detected each haplotype and the number of captured specimens that owned each haplotype in capture survey (hereafter ‘owner specimens’). In addition, for haplotype detected by Sanger sequencing using 96 specimens of Ayu, the Spearman's rank correlation test was performed to examine the relationship between the percentage of sequence reads of each haplotype in each filter replication and the number of owner specimens. All statistical analyses were performed using the statistical software R ver. 3. 2. 3 software and the significance level was set at $p \leq 0.05$.

3. Results

In Sanger sequencing of tissue-derived DNA, a total of 42 haplotypes were detected from 96 specimens of Ayu (hereafter ‘reference haplotype’). Sequences of all reference haplotypes are listed in Table S5–2. The number of detected reference haplotypes increased with the number of analyzed specimens, but the accumulation curve of detected haplotypes did not reach an asymptote even when all 96 specimens of Ayu were analyzed (Fig. 5–2a). The number of owner specimens of each

haplotype ranged from one (ID; 12–42) to 28 (ID; 01) (Fig. 5–3).

In MiSeq paired-end sequencing of eDNA, a total of 20,377,762 reads were obtained from the 333 libraries (including 300 real samples, 15 filtration negative control and 18 non-template controls). After denoising using DADA2, a total of 14,377,518 reads were remained and successfully assigned to 971 haplotypes. In filtration negative controls and non-template controls, a total of 1,539 reads were detected and finally assigned to 13 haplotypes. According to the GLM, the detection rate of each haplotype on filter or library replications showed significant positive relationships with the number of corresponding owner specimens (GLM; $p < 0.001$, Fig. 5–4a and b).

A total of 37 haplotypes out of 42 reference haplotypes were detected from total of 20 filters with 15 library replications (Fig. 5–3). Ten reference haplotypes having two owner specimens or more were detected from all 20 filters, each that had 70–100% detection rates, with an exception (ID 8, from no filters). The other 27 reference haplotypes having only one owner specimens were detected from 1–20 filters, each that had 7–100% detection rates (Fig. 5–4a and b). 934 haplotypes that did not correspond to any of the 37 reference haplotypes were also detected from 1–20 filters, each that had 7–100% detection rates (Fig. 5–4a and b).

The number of detected reference haplotypes increased with the number of analyzed filters. The 95% confidence intervals of two accumulation curves estimated using two data set including haplotypes obtained from five and fifteen library replications overlapped considerably (Fig. 5–2b). After three filters, the increase in the number of detected reference haplotypes became gentle in both of accumulation curves.

There was significant positive rank correlation between the percentage of reads of each haplotype in each filter and the number of specimens which owned corresponding haplotype in 96 captured specimens (Spearman's rank correlation test; $p < 0.01$, Fig. 5–5)

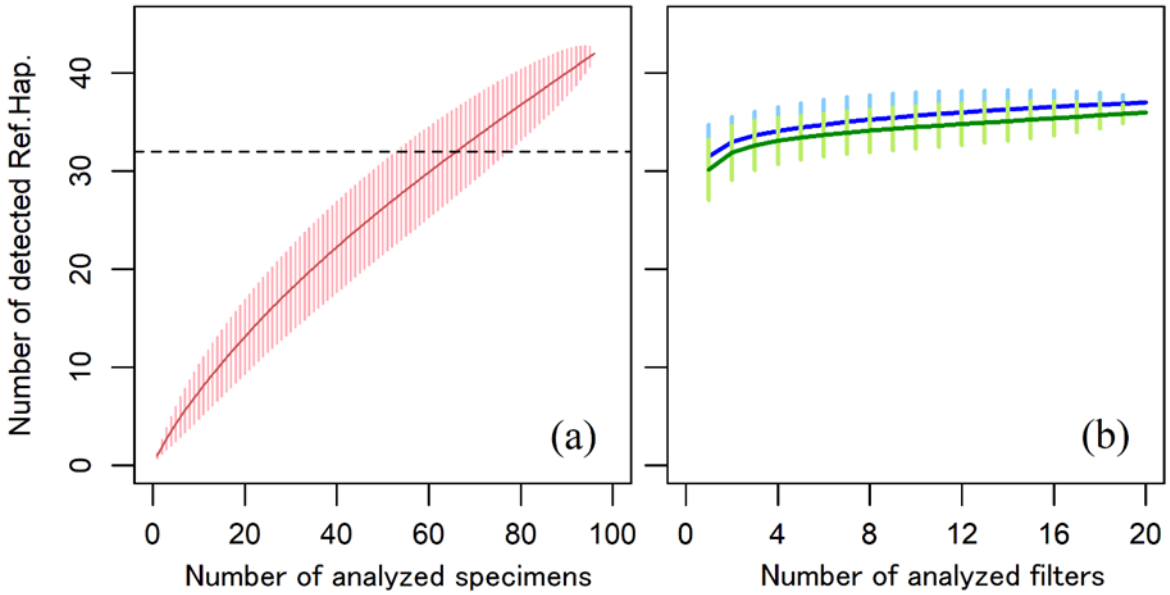

Fig. 5–2 Accumulation curves of reference haplotypes based on the number of (a) analyzed specimens in the conventional method and (b) analyzed filters in eDNA analysis. Blue and green lines represent the accumulation curve estimated using 15 (five replications in 1stPCR 5 × three replications in 2nd PCR) and five (five replications in 1stPCR 5 × one replication in 2nd PCR) library replications, respectively. Dashed line represents 32 reference haplotypes which is expected to obtain when three filters each of which has five library replications were analyzed. Vertical bars indicate 95% confidence intervals.

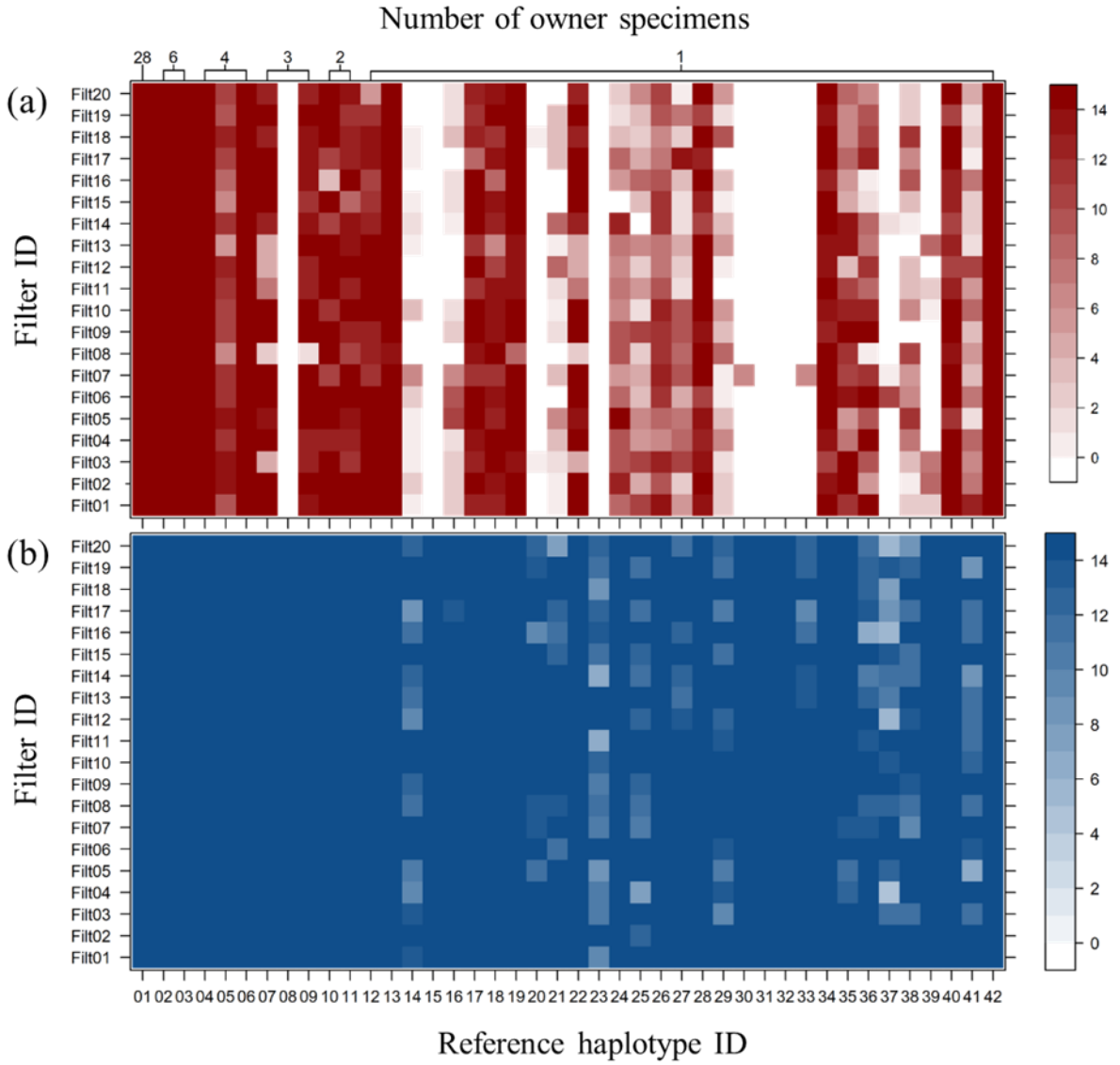

Fig. 5–3 Heatmaps depicting the number of detections among 15 library replications of each reference haplotype per filter. Each of blue and red heatmap indicate the result obtained when denoising was (a) performed or (b) not (analyzed using custom pipeline, Sato et al. 2018), respectively. Horizontal axes indicate the reference haplotype ID and number of owner specimens of each reference haplotype, respectively.

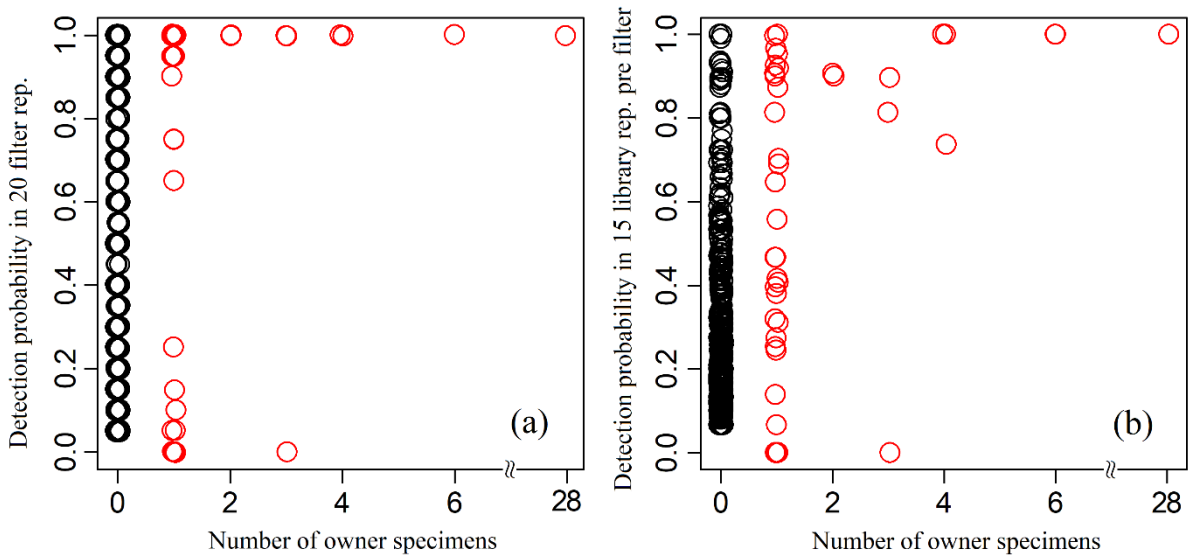

Fig. 5–4 The relationship between the number of owner specimens and (a) the detection probability in 20 filter replications, and (b) the detection probability in 15 library replications per filter. Red and black circles indicate the reference haplotypes and the haplotypes obtained from only eDNA analysis, respectively. The haplotype detected from one or more library replications out of 15 were defined as detected haplotypes from each filter. Detection rate of each haplotype out of 15 library replications in each filter was calculated using only filter data that detected its haplotype.

4. Discussion

Our study showed the effectiveness of eDNA analysis in evaluating the intraspecific diversity in wild Ayu population and demonstrated that the great potential of this approach. Understanding of intraspecific diversity is crucial for maintaining and management of fish population. The use of eDNA analysis for evaluating intraspecific diversity of population will be receiving more attention from researchers and natural resource managers.

4.1. Interpretation of intraspecific diversity data obtained with eDNA.

In conventional methods, even when all 96 captured specimens were analyzed, the accumulation curve of detected haplotypes did not reach a steady state. When this study was performed, numerous individuals of Ayu were present at the sampling site because they ascend from the Lake

Biwa to rivers from spring to autumn (Azuma 1973, Iguchi et al. 2002). The analyzed 96 specimens represent only a fraction of the Ayu population in Ado river, and it was impossible to collect and sequence all individuals of population in this study. Therefore, results of conventional method did not cover the whole intraspecific diversity of Ayu population in the sampling point; thus, the number of haplotypes detected by eDNA analysis which were also detected by the conventional method were investigated.

In eDNA analysis, a total of 934 haplotypes other than reference haplotypes were detected from total 20 filter replications. This result did not automatically imply that eDNA analysis has grater detection power than the conventional method, because the HTS data always contains numerous erroneous sequences which were derived from some sources including Taq polymerase errors or sequencing errors (Schloss et al. 2011, Coissac et al. 2012, Edgar et al. 2016). Thus, some false positive haplotypes which were derived from erroneous sequences might be included in results of eDNA analysis, despite the denoising were performed using DADA2.

Further studies are needed for evaluating overestimation of intraspecific diversity by eDNA analysis, because there was no knowledge on all haplotype present at the sampling site, as stated above.

4.2. Potential detection power of eDNA analysis compared to conventional method.

The 37 out of 42 reference haplotypes obtained from 96 Ayu specimens using Sanger sequence were detected from a total of 20 filter replications by eDNA analysis using HTS technology (Fig. 5–3). In addition, the detection rates of each haplotype among 20 filter and 15 library replications increased with the number of owner specimens of reference haplotype (Fig. 5–4a and b). Especially, except for the reference haplotype ID 08, all haplotypes owned by two or more specimens were detected from all filter replications with average 11 or more library replicates (Fig. 5–2, 5–4a and b). In some previous studies, it was suggested that the eDNA concentration has positive relationship with abundance and/or biomass of organisms (Takahara et al. 2012, Klymus et al. 2015, Doi et al. 2016, Maruyama et al. 2018). Thus, it was considered that the eDNA of real haplotypes which were

owned by more individuals would have been contained in water at higher concentration, and they are more easily amplified in PCR than rare haplotypes. Therefore, haplotypes detected from all filter and library replications by only eDNA analysis (i.e. owner specimen was zero in conventional method) might have been derived from specimens which were not sampled by the conventional method.

4.3. Relationship between the total reads and the number of owner specimens.

There was significant positive rank correlation between the percentage of reads of each haplotype on each filter and owner specimens in 96 specimens (Fig. 5–5). This result was consistent with the result of previous study using aquariums (Tsuji et al. 2018) and field water (Sigsgaard et al. 2016). Thus, it was suggested that read abundance in HTS data might reflect the quantitative relationships among haplotypes. In a recent study of eDNA metabarcoding which targeted multiple fish species, it was shown that the inclusion of internal standard DNA in eDNA sample enables simultaneous determination of the quantity and identity of eDNA of multiple species (Ushio et al. 2018). This quantitative technique for HTS technology has the potential to enable the quantitative monitoring of intraspecific diversity by eDNA analysis in future study.

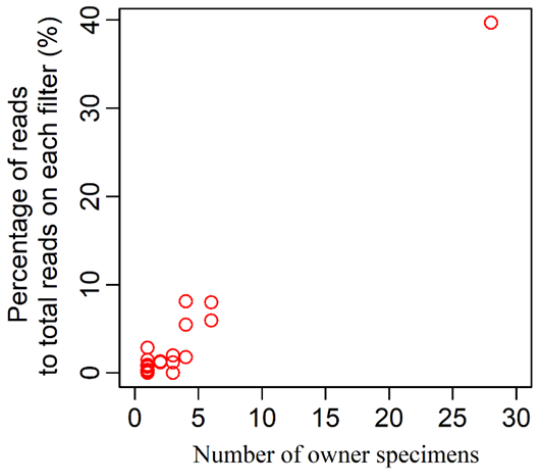

Fig. 5–5 The relationship between the number of owner specimens and the percentage of reads to total reads on each filter.

4.4. The reference haplotypes which were not detected in eDNA analysis.

Five out of 42 reference haplotypes were not detected by eDNA. In this study, it is considered that false negative haplotypes in eDNA analysis is occurred by three main reasons as follows: 1) failure of eDNA capture during sampling because of the scarcity or degradation of eDNA molecules (Evans et al. 2017), 2) failure of PCR amplification of eDNA in sample because of the scarcity or the PCR inhibition (Jane et al. 2014, Ostberg et al. 2018), 3) miss elimination during denoising of HTS data in bioinformatic analysis (Rosen et al. 2012).

To investigate the causes of false negative results of this study, all fastq files including raw reads were reanalyzed without DADA2. The base-calling errors were eliminated with the quality filtering, and the data pre-processing and dereplicating was performed using a custom pipeline described by Sato et al. (2018). As a result, all reference haplotypes were detected from all filter replications; however, detection frequency in 15 library replications was varied from 33% to 100% (Fig. 5–3). This result suggested that eDNA molecules of all reference haplotypes might be included in all water samples and were amplified at least in some library replicates of each filter replication. In addition, sample water quality was equal for all filter replications; thus, the effect of PCR bias caused by water quality was equal for all filter replications. Hence, these results suggested that the failure of eDNA amplification were caused on some PCR due to the scarcity of eDNA in each sample. Furthermore, the low read abundance which were caused by the scarcity of eDNA and the failure of PCR amplification likely to cause erroneous denoising by DADA2 (cf. Callahan et al. 2016, Tsuji et al. 2018). Therefore, in this study, it was considered that the five reference haplotypes were not detected due to the mistake during amplification and denoising which were caused by the scarcity of eDNA.

However, in reanalysis without DADA2, a total of 44,687 haplotypes other than reference haplotypes were detected from total 20 filter replications. Considering that 934 haplotypes other

than 42 reference haplotypes were detected when denoising was performed, the denoising by DADA2 eliminated 43,753/44,687 (97.9%) haplotypes that have a high probability of false positive. Therefore, while there were five false negative haplotypes, it was considered that the denoising using DADA2 greatly contributed to the improvement in detection accuracy.

4.5. Future research suggestions

Our results suggest that the detection frequency in multiple filter and library replications would become important index to select the real haplotypes from HTS data and improve the reliability of results. However, the number of detected reference haplotypes only slightly increased with the number of analyzed filters. Furthermore, the difference of the number of library replications in each filter (five or 15 replications) little affected the number of detected reference haplotypes (one or two haplotypes). Therefore, it was suggested to reduce the number of replications of filter and library from 20 to three and from 15 to five, respectively. There are some false negative haplotypes, but the result can be used as an index for estimating of intraspecific diversity. In addition, the expected result obtained from the proposed replication setting correspond to the result when about 66 specimens were analyzed by conventional method (Fig. 5–2a), and it allows us to save time and labor per site for sampling. Furthermore, the setting of threshold on detection frequency in multiple filter replicates can expect to minimize the false positive haplotype. In addition, to attain further increase in the detection accuracy, this study propose two sampling strategies in future study as follows: 1) the increasing of the filtration volume per each filter, 2) the pooling of water samples which were collected from multiple points. First, the increment of the volume of sample water expands the eDNA yield from water, and it may increase the detection probability, as suggested by Valentini et al. (2016) and Doi et al. (2017). Second, some previously studies suggested that eDNA heterogeneously distribute in water (e.g. Pilliod et al. 2013, Hunter et al. 2015, Hänfling et al. 2016). Such heterogeneous distribution of eDNA is likely to cause the detection bias, and it affect the detection probability of rare haplotype. There is some possibility of cancelling or decreasing these

risks by pooling water samples. Studying the factors affecting detection accuracy and limitations of this approach would facilitate the application of eDNA analysis for estimating intraspecific diversity in various species and field.

5. Supplementary information

Table S5–1. Primers for the second PCR. Xs indicate index sequences to identify each sample.

Table S5–2. Sequences information of all reference haplotypes.

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Chapter VI

Final Conclusion

In this study, the overall objectives were to 1) accumulate the basic knowledge about eDNA degradation that affects the results of eDNA analysis and 2) develop the new methods to broaden the applicability of eDNA analysis. To achieve these objectives, this study focused on the methods of eDNA analyses themselves for detecting aquatic macroorganisms. The trend of analysis methods used in each analysis step was revealed by reviewing most of papers in the field of eDNA analysis for macroorganisms. In addition, this study showed that the eDNA degradation rate is greatly influenced by temperature. Furthermore, two new analytical techniques for simultaneous detecting of several species and estimating of intraspecific diversity were proposed to expand the availability of eDNA analysis. The results and future challenges of this research are summarized below.

In Chapter I, entitled General Introduction, most of papers using eDNA analysis for detecting macroorganisms published in international journals by 2018 were reviewed by focusing on analysis methods. The use of several methods has been reported for each analysis step (e.g. collection, extraction, and detection), and there are many technical differences among research teams and/or studies. However, compared to the initial period when the detection of macroorganisms using environmental DNA analysis was started, there has been a tendency to converge unify to one or two methods for each analysis step for the last few years. The understanding of the current trends and the major methods used in eDNA analysis should help researchers, who has been newly engaged in eDNA study, to understand the outline of eDNA analysis. On the other hand, there are few reports on comparing of eDNA collection efficiency of the combinations of each method among analysis steps. In addition, there is no report that examines the efficiency of each method in relation to factors such as water quality and taxonomic groups. Therefore, it is recommended to accumulate

more knowledge to allow the researchers to choose efficient methods depending on the purpose, target species, and environmental condition of research field of each study.

In Chapter II, the eDNA degradation rate was examined in relation to the influence of water temperature. As a result, time-dependent degradation of eDNA was confirmed to be accelerated at higher temperature, and a non-linear model formula was constructed depending on the result. In addition, although the results of the study cannot reach final conclusions, it was suggested that bacterial abundance have influenced on eDNA degradation, and this suggestion was consistent with previous studies (Thomsen et al. 2012, Strickler et al. 2015). Thus, it was suggested that the importance of keeping sample water at low temperature between water sampling and filtration and the necessity of quick filtration because eDNA is degraded even at low temperature. Based on the above results, an on-site filtration method that filtered water sample immediately after sampling was adopted in the studies in the latter chapters to minimize eDNA degradation. The findings obtained in the chapter can be used as important information to estimate the diffusion and release rate of eDNA and are expected to contribute to the optimization of analytical method of eDNA analysis.

In Chapter III, a multiplex PCR method was newly applied for detecting several species simultaneously. Two species of Japanese medaka (*Oryzias latipes* and *O. sakaizumii*) were used as model species. In a field inhabited both medaka species, eDNA analysis with multiplex PCR was able to estimate each species habitat simultaneously. This result demonstrated that the multiplex PCR method can be applied to simultaneously detection of multiple species using eDNA, and it was suggested that multiplex PCR method would become one of the useful options in studies targeted several species. However, an examination targeting only two species had been performed in this study. Thus, to optimize this method, it would be necessary to further examine the detection power and the accuracy targeting more kinds of species. In that case, the detection power and accuracy would be increased by using dedicated PCR regent for multiplex PCR. Multiplex PCR method is a

technique that has been widely used for gene expression analysis and species identification of plants, and it has a characteristic that detects each species and/or gene by mixing some specific primer probe sets in single PCR reaction. This characteristic will allow us to simultaneous detection even when distant classification groups or different gene regions are targeted.

In Chapter IV and V, an analytical technique was proposed for eliminating false positive haplotypes derived from PCR and sequence errors, which can be considered as the biggest problem in the estimation of intraspecific diversity using eDNA analysis. The proposed technique was composed of denoising based on error models and the haplotype selection based on detection rate of each haplotype among multiple library replications. The proposed technique successfully detected 8 out of 9 known haplotypes contained in rearing water from all library replications and eliminated 99% of false positive haplotypes. Thus, it is showed that the usefulness of this technique for estimating intraspecific diversity by eDNA analysis. Field water tends to contain a variety of haplotypes at lower concentration than aquarium water; therefore, there is a possibility that increment of false negative results caused by some errors during eDNA collection and amplification due to the scarcity of DNA molecules. For this reason, in the fifth chapter which was compared the detection power of eDNA analysis with conventional method, water sample was divided onto multiple filters and library replications to examine the possibility of miss collection and amplification of scarce haplotypes. As a result, in the case of haplotypes obtained from more than 1/96 specimens by the conventional method, they were detected with almost 100% detection rate among multiple filter iterations though the detection rate was varied among library replicates. This result suggested that the false negative results in the estimation of intraspecific diversity using field water contained scarce haplotypes tended to be caused by the miss amplification during PCR rather than the miss collection. In addition, just as the fourth chapter used rearing water, the denoising based on error model successfully eliminated 98% of haplotypes that were highly likely to be false positive results because they were not detected by conventional method. Based on the above results, to eliminate

false positive haplotypes, it was finally proposed that the use of the analytical technique including the denoising based on error model and the haplotype selection based on detection rate among multiple filter replications. Furthermore, after eDNA analysis of three filters which had five library replications by eDNA analysis, the increasing the number of detected haplotypes that correspond with those of 96 specimens became gentle; thus, eDNA analysis was considered to be enough sensitive to decrease replication numbers. In two previous studies which were used eDNA analysis to estimate intraspecific diversity, the haplotypes which were not deposited in the respective sequence database were eliminated at denoising step (Sigsgaard et al. 2016, Parsons et al. 2018). However, the strategy has disadvantages that it is applicable only to confined species having enough sequence information on the database and all unknown haplotypes will be eliminated. On the other hand, the proposed analytical technique in this study solves technical problems with existing denoising strategy in previous studies and increase versatility and practicality of the estimation of intraspecific diversity using eDNA analysis, because it requires no reference sequences. In the future, to optimize the method for estimating intraspecific diversity by eDNA analysis, it is required to accumulate data by examining the detection power of this method in a different environments and taxa in more detail. In addition, by taking advantage of characteristics of eDNA analysis that are allow non-invasive and simple sampling, it is expected that eDNA analysis with proposed analytical technique will be applicable to estimate the intraspecific diversity of endangered species populations and the genetic network in broad area.

The above results of this study provide basic knowledge necessary for the future development of eDNA research and broaden the applicability to new research challenges. Knowledge on the eDNA degradation expanded in this research is important for determining the reliability of eDNA detection from collected water samples. This knowledge should be contributing to improve the reliability of results in eDNA studies by considering it. In addition, the development of new method allows us to challenge for new research areas. For example, it is expected that the eDNA analysis with multiplex

PCR will be a useful tool for further studies such as the detection of several species which have a symbiotic relationship and the monitoring of spawning activity based on the change of the abundance ratio of mitochondrial DNA and nuclear DNA. Furthermore, the estimation of intraspecific diversity using eDNA analysis will make it possible to non-invasively estimate intraspecific diversity of endangered species. In addition, it is expected that the use of eDNA analysis allows us the estimation of the genetic network in broad area because the characteristics of eDNA analysis that require only water sampling at the study site will save substantial time and labor for sampling. I hope that eDNA analysis will be further developed by continuing researches all over the world. This study will contribute to the understanding of eDNA analysis and expand the availability of eDNA analysis.

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1 **Satsuki Tsuji**, Hiroki Yamanaka (2014) On the effect of pH of water sample on the recovery rate of environmental DNA, 日本陸水学会第 79 回大会
2 **辻冴月**, 櫻井翔, 山中裕樹 (2015) 環境 DNA 分解速度の温度依存性, 第 62 回日本生態学会大会
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5 Hiroki Yamanaka, Hiromu Motozawa, **Satsuki Tsuji**, Ryohei C Miyazawa (2015) On site filtration of water samples for environmental DNA analysis to avoid DNA degradation during transportation, British Ecological Society 2015 Annual Meeting, Scotland
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Awards and Others

1 第 62 回日本生態学会鹿児島大会ポスター賞 優秀賞 (生物多様性分野)

2 第 81 回日本陸水学会沖縄大会 課題講演 2 コンビーナとして企画・運営を担当

3 第 23 回日本生態学会近畿地区会 奨励賞

4 日本学術振興会特別研究員 DC2 (2018–2019)