THE EFFECT OF STORM EVENT ON DETECTION THE SPECIES DIVERSITY OF INSECTS USING ENVIRONMENTAL DNA META-BARCODING

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ABSTRACT

Environmental DNA (eDNA) is a powerful and easy methods to discover species diversity in aquatic environments. Although various environmental factors can change eDNA analysis results, a few reports explained the effects of rains including storms have on eDNA analysis and were not enough to understand the effect of rain on eDNA detection. In this study, we investigated aquatic organism biodiversity in Tendani Creek (Hiruzen Experimental Forest, Tottori University, Maniwa, Japan) using eDNA collected before and after a storm as a strong disturbance event on river water. We collected water samples on the days before and after typhoon Talim passed on 17 September 2017. Next-generation sequencing sequenced the Cytochrome Oxidase I region for Insecta. Sequence data were identified as putative source organisms. As a result, we found a drastically higher number of operational taxonomic units (OTUs) and species after the storm than before it. This can relate to the transportation of organic matter from the land by rainwater and to the resuspension of sedimented organic matter from riverbed disturbance. On the other hand, the detection rate of aquatic species decreased after the storm. This meant that rainwater diluted DNA from aquatic species. Although the post-storm eDNA can reflect biodiversity not only in water but also on land, the pre-storm eDNA is suitable for biological monitoring in the aquatic environment.

Keywords: aquatic insect, typhoon, monitoring, environmental DNA, DNA barcoding

1. INTRODUCTION

Biodiversity monitoring has been conducted for environmental assessments in aquatic environments based on information about macroinvertebrate communities. Macroinvertebrates, including aquatic insects, are suitable bioindicators of artificial impacts (e.g., flood control, water quality, and habitat loss) because of low mobility (e.g., hundreds of meters to tens of kilometers) and more information (i.e., number of species, number of individuals) compared to other taxa. In general, monitored information has been investigated by the method, combining field sampling and morphological identification.

The monitoring method is widely used but has several problems. For field sampling, survey sites and frequency are hard to increase because of limited human resources. Then, collected organisms depend on field workers' sampling skills, and field sampling can disturb the habitat of rare species, such as endemic and endangered species. For morphological identification of macroinvertebrates, complicated-classification keys lead to misidentification, and classification levels can be rough at the level of the genus/family frequently due to many undescribed species. Moreover, identification takes a long time because tens or hundreds of macroinvertebrates are collected from one field frequently.

In recent years, environmental DNA (eDNA) has become a powerful and easy tool for revealing the species diversity in aquatic environments. The eDNA is defined as the DNA that exists in environments such as water, soil, and air (Taberlet et al. 2012). Aquatic organisms seem to release DNA with their cells (e.g., shedding skins and excrement) into outside environments (Taberlet et al. 2012; Thomsen and Willerrslev 2015). Because the eDNA contains various organic matters originating from organisms that live there, we can examine the community structure of inhabiting organisms only by water sampling and standard DNA analysis. Previous studies reported the ability to use eDNA for biomonitoring in aquatic environments (for fish: Takahara et al. 2013; Miya et al. 2015; Kelly et al. 2014; for salamanders: Fukumoto et al. 2015; for aquatic insects: Deiner et al. 2016; for mammals; Ushio et al. 2017).

DNA barcoding is a method used to identify organisms based on DNA sequences (Kumar et al. 2015). This method is known as easy identification and is employed for biodiversity monitoring using bulk DNA (Serrana et al. 2018; Serrana et al. 2019), gut contents (Jo et al., 2016), and eDNA (Miya et al. 2015; Kelly et al. 2014; Deiner et al. 2016; Ushio et al. 2017). First of all, in the DNA barcoding, DNA was amplified by PCR by universal primers, which were designed for target taxa, e.g., for fish, MiFish (Miya et al. 2015); for mammal, MiMammal (Ushio et al. 2017); for Insecta, BF1&BR2 (Elbrecht and Florian 2017). Next, the PCR products read DNA sequence with a high-throughput/next-generation sequencer (NGS), which is an emerging and growing technology for reading a massive number of DNA sequences in parallel (e.g., Illumina MiSeq system, 44-55M reads), comparing with the Sanger sequencing method. Then, the origin organisms of each DNA sequence are estimated from searching DNA databases, e.g., the National Center for Biotechnology Information (NCBI), the Barcode of Life Data System (BOLD). Finally, we can construct the species list of inhabiting organisms based on the estimated original organisms.

Several studies reported that various environmental factors could change the condition of eDNA in water (Tréguier et al. 2014, Barnes et al. 2014, Piaggio et al. 2014, Strickler and Goldberg 2015, Jane et al. 2015). DNA can decompose due to both abiotic factors (e.g., UV, acidic environment, high temperature, sediment, river flow) and biotic ones (e.g., microbial community, extracellular enzyme). The detection rate of specific organisms from eDNA was also affected by their activities (i.e., spawning seasons and habitats). However, less is known about the effects of storm events on eDNA barcoding. Storm events such as typhoons frequently cause heavy rain. To be compared with normal rain, storm rain can clearly change the composition of suspended organic matter through inflow from land and the disturbance of river beds. The effects of storm events on eDNA barcoding must be investigated if effective biomonitoring is to be implemented.

In this study, we investigated the effect of a storm event on eDNA barcoding analysis. Water was sampled at four sites along the Tendani River (Hiruzen Experimental Forest, Tottori University, Maniwa, Japan) on the 16th and 18th of September 2017. Typhoon Talim passed through this area on the 17th of September 2017. The Cytochrome Oxidase I (COI) region targeted on Insecta class was sequenced by NGS Miseq (Illumina). Sequence reads were classified into putative original organisms. Finally, we discussed the composition of organisms that were identified from eDNA before and after the storm event.

2. METHODS

2-1 Study sites

The study field was located in Tendani Creek in the western part of the Hiruzen Experimental Forest of Tottori University (Maniwa, Japan, Fig. 1). The environmental conditions in the field were described by Haga et al. (2017). In summary, Tendani Creek is a headwater of the Asahi River. Study sites are perennial third-order streams and show the step-pool sequences. The river bed is mainly composed of gravel and cobble. The forests are predominantly composed of several types of oaks (e.g., Quercus spp.) and plantation trees (e.g., Cryptomeria japonica and Chamaecyparis obtusa).

Four sampling sites (SH, SS, SM, and SD, Fig. 1) were located in the creek. Three of the four sites (i.e., SH, SM, SD) were along the main stream. Catchment areas were 5.93ha, 41ha, and 118ha, respectively. The SS was for the sampling site of spring water generated at the valley bottom of the lowest part of an unchanneled catchment (catchment size 3.2ha).

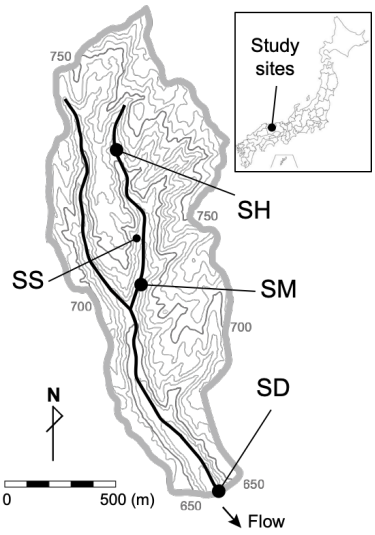

Fig1. Location of study sites.

2-2 Water sampling and filtration

Water sampling was performed on two days (i.e., the 16th morning and 18th morning in September 2017) before and after a storm. On 17th September 2017, Typhoon Talim passed over this field. Figure 2 shows hourly precipitation measured by the Automated Meteorological Data Acquisition System (AMeDAS, the Japan Meteorological Agency) in Kaminagata (Okayama, Japan), which is about 10 km away from the study field. Heavy rain started on the afternoon of 17th September and ended early in the morning on 18th September. The maximum precipitation was about 30 mm/h.

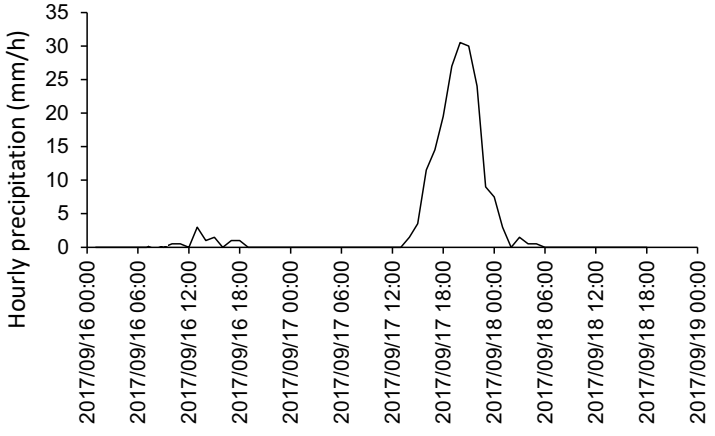

Fig2. Hourly precipitation in Kaminagata (Okayama, Japan)

Water sampling before the storm was conducted at the time of ordinary discharge. However, water collection after the storm was performed when the water level was higher than the ordinary discharge. We carried water with plastic bottles to our laboratory. 300 ml of river water was filtered using a membrane filter (ADVANTEC, material Mixes Cellulose Ester Membranes, pore size = 0.2 μm). The filters were preserved in a 2 ml tube at -20°C.

2-3 DNA Extraction

DNA from each filter was extracted by phenol-chloroform extraction and isopropanol precipitation. First, each filter was shredded and put in a 2 ml tube using flame-sterilized scissors and tweezers. The fragments were mixed with 650 μl of HMW buffer (final concentration; 0.1M Tris, 0.1M EDTA, 0.75M NaCl), 6.5 μl of proteinase K (Qiagen), and 6.5μl of 10% SDS. Then, the tube was incubated for two hours in a water bath for protein decomposition. Next, we performed the phenol-chloroform extraction. 650 μl of TE saturated phenol was added into the tube and mixed well. The tube was centrifuged (10000 x g, 10 minutes, room temperature) and the upper layer was transferred to a new 1.5 ml tube. We add 225 μl of TE saturated phenol and CIA (Chloroform: Isoamylalkohhol = 24:1) into the tube and mixed well. We performed centrifugation and transferring under the same conditions again, then isopropanol precipitation was performed. We mixed the extraction with 70 μl of 3M sodium acetate (pH 5.2) and 490 μl of isopropanol preserved at -20°C. It was centrifuged (10000 x g, 15 min, 0–4 °C), and isopropanol was discarded. Then, 1 ml of 70% ethanol was added into the tube and centrifuged in the same condition. The 70% ethanol was discarded, and the tube was dried. The DNA was resuspended using 200μl of a TE buffer (pH 8.0). Then, we purified the DNA using the One-Step PCR Inhibitor Removal Kit (Zymo Research) because this DNA still had PCR inhibitors. Finally, the extracted DNA was stored at -20°C.

2-4 PCR

We performed PCR amplification targeted on the COI (COI) region of mitochondrial DNA in insect class. Because the region has been employed for DNA taxonomic analyses of animals, sequences read from many types of animals were registered into DNA databases. The reaction solution composed of 15.5μL of PCR Grade Water, 4μL 25 mM dNTP (TaKaRa), 10μL of 5x Phusion GC buffer (New England), 10μM forward and reverse primer (BF1 and BR2 primer sets (Elbrecht and Florian 2017) with 1st PCR tails for Illumina (sequencing system) 5μL, 10% Tween20 5μL, a total of 0.5μL Phusion high-fidelity DNA polymerase (New England) and 5μL of 10-time diluted DNA were mixed. PCR steps were conducted by PCR Thermal Cycler Dice (TaKaRa). The PCR cycling conditions were 95°C for 3 minutes, followed by 40 cycles 95°C for 30 seconds, followed by 50°C for 30 seconds and 72°C for 45 seconds, then followed by extension at 72°C for 5 minutes. Finally, the PCR products were preserved at 8°C. All PCR products were purified using FastGene Gel/PCR extraction kit (Nippon Genetics) and were confirmed for purification quality using Agilent 4200 TapeStation (Agilent).

2-5 Next-generation sequencing and data analysis

The PCR products were sequenced from both ends using the MiSeq system (Illumina) in Bioengineering lab (Kanagawa, Japan). Sequence quality was confirmed using FastQC v0.11 (Andrew 2010). To control sequence read quality, we employed Trimmomatic v0.39 (Bolger et al. 2014) to trim the primer region and low-quality end (i.e., less than 20) as well as removing low-quality reads (i.e., average sequence quality < 15) and short reads (i.e., less than 50 bp). The remaining reads were joined by PEAR v0.98 (Zhang et al. 2014) using default settings. Operational taxonomic units (OTUs) were clustered with 97% homology using Claident v0.2 (Tanabe and Toju 2013) on each site. All OTUs were searched for possible original organisms using the Basic Local Alignment Search Tool (BLAST) (Edgar 2010) using the nt database for all registered DNA (National Center for Biotechnology Information, NCBI) and Insecta DNA database extracted from NCBI on 16th January 2020. Finally, we removed OTUs with short matching lengths (i.e., less than 150bp) and low identification rates (i.e., less than 90%). OTUs that were assigned to the order Insecta were sorted to family levels.

3. RESULTS

3.1 Number of reads and OTUs

We gained 0.8x106 sequenced reads in total. The reads that passed quality filtering were 0.2x106 in total. The joined reads were 0.2x106 in total. The number of OTUs was 24 to 4,986 in each site (Fig. 3). Three of four sites (i.e., SS, SM, SD) showed a higher number of OTUs after the storm than before it. In particular, OTUs drastically increased after the storm in SS. SH showed almost the same number of OTUs before and after the storm.

3.2 Detected taxa

DNA barcoding using the nt database (i.e., database of all registered nucleotides) from NCBI detected 17 to 804 taxa in a site (Fig. 4). All sites showed a higher number of taxa after the storm than before it. Most reads were classified as other taxa such as bacteria and algae.

We found several insect taxa; five orders (i.e., Diptera, Hymenoptera, Lepidoptera, Plecoptera, and Trichoptera), 13 families (i.e., eight in Diptera, two in Lepidoptera, one in Hymenoptera, one in Plecoptera, and one in Trichoptera), and 17 genera (11 in Diptera, two each in Plecoptera and Lepidoptera, and one each in Hymenoptera and Trichoptera) (Table 1). Focused on Insecta families, five families were detected both before and after the storm. On the other hand, the other eight families were found either before or after the storm. Further, three of four families that were found only after the storm lived on land. Two of four families that were found only before the storm (Ceratopogonidae in Diptera and Odontoceridae in Trichoptera) were aquatic species.

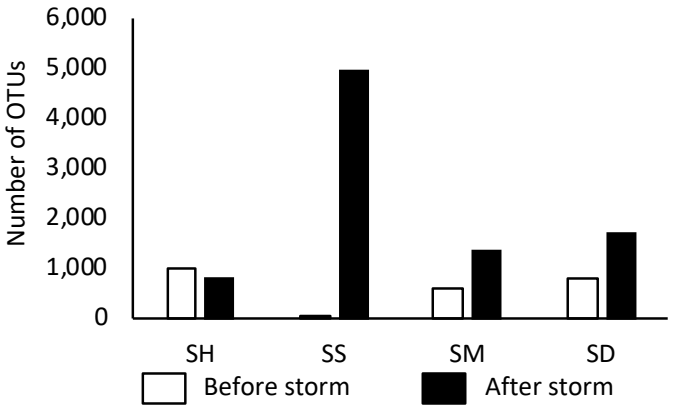

Fig3. Number of OUTs in each site.

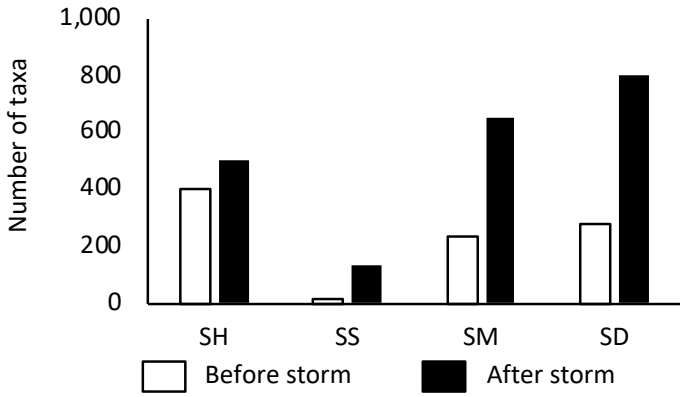

Fig4. Number of detected taxa in each site.

4. DISCUSSIONS

We found a higher number of OTUs and taxa from eDNA in most study sites, and three inland species were detected only after the storm. These changes seemed to be caused by two different factors. The first is the transportation of organic matter by storm. Rainfall on land flows into the river throughout the surface and underground. At that time, rainwater can also flash and carry organic matter from the land into the river. Therefore, river water after the storm contained both DNA in water and land, and inland organisms could be found by eDNA. The second factor is the disturbance of sediments on the river bottom. When a storm passes, rivers often show higher water volume and flow velocity. These conditions disturb the riverbed, and sedimented organic matter can be resuspended into the river. Then, eDNA in the river after the storm can contain DNA originating from organisms inhabiting not only the water but also the riverbed and underground.

Rainfall also possibly interrupted the detection of aquatic species. In this study, we could not detect two aquatic insects after the storm, although they were found beforehand. In general, water volume in rivers increases after storms. This can cause dilution of the DNA that existed before a storm. Due to the low concentration of DNA, it can be hard to find the NGS amplicon sequence. Therefore, we could not find several aquatic species. This suggests that it is not suitable to perform DNA analysis targeted at aquatic species after storms. However, it is still unclear how much precipitation will affect eDNA analysis and the range of precipitation showing no effect. These points well are the next studies in the future.

Table 1 Detected taxa before/after storm. 0 = non-detected, 1 = detected.

Order	Family	Genus	Before	After
Diptera	Anthomyiidae		0	1
	Ceratopogonidae	Atrichopogon	1	0
	Chironomidae	Corynoneura, Chironomus, Micropsectra, Orthocladius, Paramerina, Parametriocnemus	1	1
	Drosophilidae	Mycodrosophila	1	1
	Empididae		0	1
	Hybotidae	Bicellaria	1	0
	Simuliidae	Simulium	1	1
	Syrphidae	Sphegina	0	1
Hymenoptera	Apidae	Epeolus	1	1
Lepidoptera	Epicopeiidae	Epicopeia	0	1
	Notodontidae	Notodontinae	1	0
Plecoptera	Nemouridae	Amphinemura, Nemoura	1	1
Trichoptera	Odontoceridae	Odontoceridae	1	0

Although our target taxa were Insecta species, we also detected broad taxa such as bacteria and algae. It suggested that PCR primer adapted not only Insecta but also other organisms, including microorganisms. This phenomenon was reported by other primers on the COI region (Deiner et al. 2014). The lack of suitable PCR primers is a significant problem for eDNA barcoding that targets aquatic insects. Therefore, it is necessary for efficient biodiversity monitoring using eDNA to develop new primers to amplify only insects.

5. CONCLUSIONS

We examined the effect of the typhoon Talim in 2017 on the result of eDNA barcoding on the COI region for Insecta in the Tendani River (Hiruzen Experimental Forest, Tottori University, Maniwa, Japan).

1) Both OTUs and species after the storm showed a higher number than before the storm. This suggests that the eDNA after storms can reflect not only aquatic environments but also the surrounding environment because the storm transports and resuspends organic matter into the river water.

2) Less aquatic species are found after the storm than before. This meant that DNA from aquatic species was diluted by rainwater, which is difficult to detect because of low concentration. For the monitoring of aquatic environments, it should be performed at the time of ordinal flow.

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