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Detection of herbivory: eDNA detection from feeding marks on leaves

Aoi Kudoh[1] | Toshifumi Minamoto[2] | Satoshi Yamamoto[3]

[1]Faculty of Agriculture, Kyoto University, Kyoto, Japan

[2]Graduate School of Human Development and Environment, Kobe University, Kobe, Japan

[3]Department of Zoology, Graduate School of Science, Kyoto University, Kyoto, Japan

Correspondence
Satoshi Yamamoto, Department of Zoology, Graduate School of Science, Kyoto University, Kitashirakawa-oiwake-cho, Sakyo, Kyoto 606-8502, Japan.
Email: s_yamamoto@terra.zool.kyoto-u.ac.jp

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Abstract

Many techniques have been developed to investigate the interactions between plants and herbivorous insects in natural environments and are generally used to determine either (a) which plant species are eaten by a specific herbivorous insect or (b) which herbivorous insect species are herbivores of a specific plant. The former problem is usually addressed by the direct observation of feeding and microscopic observation of gut contents and excrements, as well as the application of DNA-barcoding techniques. However, the latter problem has typically been addressed using time-consuming methods, such as direct observation and rearing. Therefore, more efficient techniques are needed for identifying and quantifying the interactions of plants with herbivorous insects. The present study demonstrates that the environmental DNA (eDNA) of herbivorous insects can be recovered from leaves with external foliage feeding marks. Mitochondrial DNA fragments of herbivorous insects were detected from insect-exposed leaves using primer sets that amplified the DNA of target species. The amplification rate of the herbivorous insect DNA was positively associated with the rim length of feeding marks, which suggests that most of the insect DNA came from the feeding marks. Additionally, we showed that this method has the potential to detect eDNA from field-collected leaves. This time-efficient approach will contribute to the detection of plant–insect herbivore interactions.

KEYWORDS
herbivory, insect, larva, leaf, real-time PCR, saliva

1 | INTRODUCTION

Herbivores have a significant impact on plants, consuming more than 10% of their annual primary productivity on average (Cebrian, 1999; Coupe & Cahill, 2003; Cyr & Pace, 1993; McNaughton, Oesterheld, Frank, & Williams, 1989). Elucidating interactions between plants and herbivores is therefore ecologically and agriculturally important. In particular, many researchers have focused on the interactions between plants and herbivorous insects (Després, David, & Gallet, 2007; Farrar, Barbour, & Kennedy, 1989; Gatehouse, 2002). Various factors such as species preference, the presence of other organisms, and abiotic environment can modify these interactions. However, quantitative observations of herbivory under natural conditions are not generally frequent enough to dissect these complex factors because they are generally difficult and require great effort. Therefore, the development of a method that

allows efficient investigation after insect feeding behavior is necessary to understand interactions between plants and insect herbivores in a natural environment. Such a method could help elucidate ecosystem structure, manage damage to cultivated crops, or conserve endangered species.

Investigations of such interactions between plants and herbivorous insects in natural conditions generally aim to determine either (a) which plant species are eaten by a specific herbivorous insect or (b) which herbivorous insect species are herbivores of a specific plant. The former is usually addressed by the direct observation of feeding and the microscopic observation of gut contents and excrements (Symondson, 2002; Yoshioka, Kadoya, Suda, & Washitani, 2010). In addition, recent studies have reported methods for recovering plant DNA from the digestive tracts and excrement of insects and from honey (Hawkins et al., 2015; Kishimoto-Yamada et al., 2013; Navarro, Jurado-Rivera, Gómez-Zurita, Lyal, & Vogler, 2010; Symondson, 2002; Valentini et al., 2009; Yamamoto & Uchida, 2018). Such approaches facilitate the efficient surveillance of difficult-to-observe areas, such as tropical forest canopies, and enable large-scale surveys of insects' plant choice (Kishimoto-Yamada et al., 2013).

Meanwhile, studies that aim to determine which insect species are herbivores of a specific plant often rely on direct observation and rearing (Ødegaard, Diserud, & Østbye, 2005; Weiblen, Webb, Novotny, Basset, & Miller, 2006). However, great efforts must be taken to observe insect feeding directly in natural conditions (Jurado-Rivera, Vogler, Reid, Petitpierre, & Gómez-Zurita, 2008), and the feeding behavior of captive animals is not necessarily representative of behavior under natural conditions (Cree, Lyon, Cartland-Shaw, & Tyrrell, 1999). Moreover, it is generally difficult to investigate the herbivores of a specific plant species at large scales based on these methods, and more effort is necessary to combine these methods to avoid over- or underestimates of interspecies interaction. Therefore, more efficient techniques are needed for identifying and quantifying the interactions of herbivorous insects with plants.

One potentially useful method is the recovery of environmental DNA (eDNA) from the external foliar feeding marks left by herbivorous insects. Indeed, several recent studies have reported that arthropod eDNA can be detected from parts of terrestrial plants (Bittleston, Baker, Strominger, Pringle, & Pierce, 2016; Derocles, Evans, Nichols, Evans, & Lunt, 2015; Thomsen & Sigsgaard, 2019). In addition, other previous studies have reported that some herbivorous insects leave secretions on leaves (Musser et al., 2002; Takai et al., 2018), and it is plausible that DNA could be recovered from such secretions and used to identify herbivores.

Accordingly, the aim of the present study was to determine whether the eDNA of herbivorous insects could be detected from leaves with external foliar feeding marks using PCR amplification. The target species were silkworm (*Bombyx mori* L.) and the small copper butterfly (*Lycaena phlaeas daimio* Seitz). White mulberry (*Morus alba* L.) leaves with fresh feeding marks by *B. mori* were obtained using controlled feeding experiments, whereas Japanese dock (*Rumex japonicus* Houtt.) and sorrel (*Rumex acetosa* L.) leaves with feeding marks by *L. phlaeas* were collected from the field. The present study also investigated whether the insect DNA came from the feeding marks by comparing the sizes of the feeding marks to the number of successful PCR amplifications.

FIGURE 1 Equipment used to collect leaves with *Bombyx mori* feeding marks

2 | MATERIALS AND METHODS

2.1 | eDNA sampling in a controlled feeding experiment

Our experiment was conducted using *B. mori*, a model Lepidopteran, and *M. alba*, a primary host plant, under laboratory-controlled conditions (the controlled feeding experiment, hereafter). Leaves with feeding marks from fifth-instar larvae of *B. mori* were used as this instar creates larger feeding marks than younger instars. Third-instar larvae (purchased from Kougensha Co., Ltd.) were reared at 25°C under a 12-hr photoperiod and fed an artificial diet (Kougensha) for 19–23 days in order to obtain fifth-instar larvae. Fresh leaves of *M. alba* were harvested from the Graduate School of Science Botanical Gardens of Kyoto University and provided to these larvae for the entire experimental period. Prior to providing the leaves, each leaf was washed for 20 s with tap water and rinsed with autoclaved water to remove potential DNA contaminants from the leaf surface. The washed leaves were wiped with clean paper, cut into ~6 × 6 cm pieces, and then clipped onto a device (Figure 1). Individual larvae were transferred to the leaves using clean chopsticks and allowed to

feed for 30 min, after which the larvae and leaf pieces were retrieved. The feeding experiment was performed over 3 days, and groups of 9, 9, and 2 individuals were randomly selected each day from 21 larvae; we therefore performed 20 feeding trials. Additionally, one negative control trial, in which *B. mori* larvae were not introduced, was included each day to assess possible sample contamination (i.e., sampling blank). After each trial, the experimental leaves were rinsed in 50-ml centrifuge tubes containing 15 ml of sterile water to collect eDNA, and then, the tubes were supplemented with 1.5 ml of 3 M sodium acetate (pH 5.2) and 33 ml of absolute ethanol, mixed, and then stored at −20°C until DNA extraction. The devices, scissors, and chopsticks were bleached between each feeding experiment. Finally, the presence of feeding marks on the collected leaves was assessed, and the rim length of each feeding mark was measured from photographs using ImageJ software (version 1.52a; National Institute of Health; http://imagej.nih.gov/ij).

2.2 | eDNA sampling in natural conditions

The butterfly *L. phlaeas daimio* (Lycaenidae) was used as the focal species of the field experiments. The species is one of the world's most widespread temperate-zone butterfly species (León-Cortés, Cowley, & Thomas, 2000). Only one subspecies of *L. phlaeas* occurs in Japan (Shirouzu, 2006), where adult butterflies are observed during spring and summer, and the spring morph overwinters in the larval stage (Endo, Maruyama, & Sasaki, 1985). As larvae start feeding on their species host plants (*R. japonicus* and *R. acetosa*) before the start of intense feeding by principal herbivorous insects (such as chrysomelid beetles; Suzuki, 1989), the majority of feeding marks on those plants are expected to be generated by *L. phlaeas daimio* in early spring.

Leaves with feeding marks were collected from two *R. japonicus* and 17 *R. acetosa* that were found on paths between rice paddies in Shiga prefecture, Japan (34°945′–34°921′ N, 136°200′–136°241′ E) in early spring (March 3, 2019). Clean rubber gloves were used during leaf collection, and new rubber gloves were used when collecting leaves from each plant to minimize cross-contamination. A negative control leaf, without feeding marks, was also collected to assess contamination during sampling (i.e., sampling blank). After confirming that no excrement was present, each leaf was transferred to an individual 50-ml centrifuge tube containing 7.5 ml of water and rinsed by shaking the tube gently 20 times to collect eDNA, and then, the leaves were removed from the tubes and discarded. Subsequently, 750 µl of sodium acetate (3 M, pH 5.2) and 16.7 ml of absolute ethanol was added to each tube and mixed, and the resulting samples were stored at −20°C until DNA extraction.

2.3 | DNA extraction

DNA was extracted from the samples in a dedicated room that was separate from the locations where PCR and the extraction of DNA from bio-tissue samples were performed. Following the methods of previous eDNA studies (Ficetola, Miaud, Pompanon, & Taberlet, 2008), the samples were centrifuged (10,000 *g*, 30 min, 4°C), and after discarding the supernatant, the resulting pellets were dried by leaving tubes open and resuspended in 200 µl sterile water. DNA was then extracted from each sample using a DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's instructions. Finally, the resulting eDNA was eluted in 110 µl of AE buffer and was stored at −20°C until DNA amplification. To ensure that no contamination was introduced through either the extraction reagents or procedure, a control sample, which did not contain eDNA, was included in each batch of DNA extraction (i.e., extraction blank).

2.4 | Primer design

A primer pair (5′-ATAGAGGAAGATCCGTAGATC-3′ and 5′-CCCTACAGCTCATACAAATAAG-3′) was designed to amplify a 112-bp segment of the *B. mori* mitochondrial cytochrome oxidase subunit 1 (*cox1*) gene. Similarly, both a primer pair (5′-AATTTGAGCAGGAATAGTAGGAACC-3′ and 5′-TGAAGGCATGTGCAGTTACAATAG-3′) and a TaqMan probe (5′-FAM-CGTCTTGAATTAGGTACTCCAGGAT-NFQ-MGB-3′) were designed to amplify and detect a 73-bp segment of the *L. phlaeas daimio cox1* gene. The primers and probe were designed using DNA sequences from the target species (*B. mori* and *L. phlaeas daimio*) and their related species/subspecies, which were obtained from the National Center for Biotechnology Information (NCBI) GenBank database. Then, the specificity of the primers and probe was checked using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) against the subset of the nr database that contained sequences from the *Insecta* (Taxonomy ID: 50,557) and the target and related species that Taxonomy ID were listed in Table S1. The related species are selected following the previous studies (Liu, Gu, & Wang, 2017; van Dorp, 2004). In addition, these specific primers and probe sets were tested by performing PCR or qPCR using the target and one or two related species that potentially occur at the study site. For the testing of *B. mori* primers, one DNA sample extracted from the body of *B. mori* was used as the target species, and two DNA samples extracted from the body of a wild silkmoth (*Bombyx mandarina* Moore) were used as the related species. The adult stage of *B. mandarina* was collected at the riverside of the Kizu River, Joyo, Kyoto, Japan, on 6 November 2017. For the testing of *L. phlaeas daimio* primers and probe, a DNA sample extracted from the body of *L. phlaeas daimio* was used as the target species, and DNA samples extracted from the bodies of a pale grass blue butterfly (*Zizeeria maha* Kollar) and a short-tailed blue butterfly (*Everes argiades* Pallas) were used as the related species. The adult stages of *Z. maha* and *E. argiades* were collected at the Graduate School of Science Botanical Gardens of Kyoto University on 2 and 20 August 2019. In each sample, DNA concentrations were adjusted to 5.0 pg/µl, 5.0×10^{-1} pg/µl, 5.0×10^{-2} pg/µl, and 5.0×10^{-3} pg/µl to reproduce expected range of eDNA concentration. PCR was performed

under the same conditions used in eDNA detection from feeding marks (see Section 2.5.1), and qPCR conditions were as defined in Section 2.5.2. Eight replicates were included in each sample and concentration.

2.5 | DNA amplification and data analysis

2.5.1 | Amplification and analysis of sequences from *Bombyx mori* feeding marks

PCR was performed using a GeneAtlas G02 Thermal Cycler (ASTEC), and each 20-µl reaction contained 14.9 µl of water, 2 µl of 10 × Ex Taq Buffer (TaKaRa), 1.6 µl of dNTP mixture (2.5 mM each nucleotide; TaKaRa), 0.2 µl of each 10-µM primer, 0.1 µl of Ex Taq HS (TaKaRa), and 1 µl of eDNA solution from the experimental leaves. Eight replicate amplifications were performed for each sample. The thermal cycle profile was as follows: 98°C for 2 min; 50 cycles of 98°C for 10 s, 56°C for 30 s, and 72°C for 20 s; and a final holding step at 4°C. In the preliminary experiment, some thermal profiles (45 and 50 cycles and 52–60.5°C for annealing temperatures) were tested using several eDNA samples and the positive control DNA that was extracted from the body of *B. mori*. Within the condition that the eDNA samples were amplified, the lowest cycle number (i.e., 50) and the highest annealing temperature (i.e., 56°C) were determined to suppress the amplification of nontarget species. In each batch of PCRs, eight negative control replicates (i.e., pure water) and eight positive control replicates (i.e., DNA extracted from the body of *B. mori*) were included to ensure that no contamination was introduced through either the PCR reagents or procedure and that the PCR procedure was able to amplify the fragment of interest. The resulting PCR products were visualized using electrophoresis on 1.5% agarose gels and purified using the Wizard SV Gel and PCR clean-up system (Promega). Then, the purified DNA was subject to reactions using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). In this reaction, we used the same forward and reverse primer with PCR amplification and then sequenced at Fasmac Co., Ltd by following supplier's protocol. Finally, the obtained sequences were confirmed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

We calculated Spearman's rank correlation in R version 3.4.0 (R Core Team, 2017) to assess the relationship between the number of positive PCR amplifications and the rim length of the feeding marks. In addition, to visualize the relationship, we generated a scatter diagram using the plot function in R.

2.5.2 | | Amplification and analysis of sequences from *Lycaena phlaeas daimio* feeding marks

For the detection of *L. phlaeas daimio* DNA, TaqMan qPCR was used to increase detection sensitivity and specificity. TaqMan qPCR was performed using a LightCycler 96 (Roche), and each 20-µl reaction contained 7 µl water, 10 µl 2 × TaqPath qPCR Master Mix, CG (Thermo Fisher Scientific), 0.18 µl each 100-µM primer, 0.25 µl 10-µM probe, and 2 µl eDNA solution. Eight replicate amplifications were performed for each sample. The thermal cycle profile was as follows: 50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 15 s and 60°C for 60 s; and 37°C for 30 s. In each batch of qPCRs, eight negative control replicates (i.e., amplification blank consisting of pure water) were included to ensure that no contamination was introduced through either the qPCR reagents or procedure, and the resulting qPCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Then, according to the manufacturer's protocol, the purified DNA was subject to reactions using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) with each forward and reverse primer that were used for the qPCR, and sequenced. Finally, the obtained sequences were confirmed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3 | RESULTS

3.1 | Primers and probe specificity test

The Primer-BLAST analysis indicated that the primers were highly specific (Table S1). The *B. mori* primers matched 76 of the 79 templates of *B. mori*, eight of the 256 templates of *B. mandarina*, one of the six templates of mulberry white caterpillar (*Rondotia menciana* Moore), and none of the other two related species. The *L. phlaeas daimio* primer matched 100 of the 104 templates of *L. phlaeas daimio*, and none of the 14 related species. The *B. mori* PCR assay and *L. phlaeas daimio* qPCR assay indicated that the primers and probe were specific when the target DNA was found in extremely low concentrations such as is found in eDNA samples (Table S1). For the test of the *B. mori* primers, *B. mori* samples were amplified in all eight replicates for 5.0 pg/µl, eight replicates for 5.0×10^{-1} pg/µl, eight replicates for 5.0×10^{-2} pg/µl, and five replicates for 5.0×10^{-3} pg/µl. In contrast, *B. mandarina* samples were amplified in eight, six, three to four, and one to two replicates for each concentration. For the *L. phlaeas daimio* primers and probe test, *L. phlaeas daimio* samples were amplified in all eight replicates for 5.0 pg/µl, eight replicates for 5.0×10^{-1} pg/µl, eight replicates for 5.0×10^{-2} pg/µl, and five replicates for 5.0×10^{-3} pg/µl. Additionally, *E. argiades* samples were amplified in four, zero, one, and one replicates for each concentration, while *Z. maha* samples were amplified in zero, zero, two, and zero replicates for each concentration. However, when the eDNA samples obtained from leaves with feeding marks were sequenced, the samples were always identified as target species.

3.2 | Detection of *Bombyx mori* eDNA (the controlled feeding experiment)

The 20 feeding experiments yielded 13 and seven leaves with and without *B. mori* feeding marks, respectively, and positive PCR

amplification was achieved for nine of the 13 leaves with feeding marks and for two of the seven leaves without feeding marks. In addition, the amplified DNA fragments were sequenced and confirmed as partial *cox1* sequences of *B. mori* by performing alignments with reference sequences (Table S2). Of the eight PCR replicates, the mean numbers of PCR replicates successfully amplified were 4.3 for leaves with feeding marks and 1.5 for leaves without feeding marks (Table 1). The number of successful amplifications was positively correlated with the rim length of feeding marks: Spearman rank correlation coefficient was 0.510 (p-value <.05; Figure 2). For blank samples, one of eight replicates for one of the three sampling blanks resulted in the positive amplification of *B. mori* DNA, whereas no amplification was observed for either the extraction or amplification blank.

3.3 | Detection of *Lycaena phlaeas daimio* eDNA (natural conditions)

Lycaena phlaeas daimio eDNA was amplified from one of the 19 leaves with feeding marks and was successfully amplified in all eight replicates (Table 1). Furthermore, the amplified fragment was confirmed as a *cox1* sequence of *L. phlaeas daimio* using NCBI BLAST (Table S2), and no amplification was observed for the sampling, extraction, or amplification blank.

4 | DISCUSSION

In the present study, herbivorous insect eDNA was successfully amplified from the external feeding marks on leaf samples obtained from feeding experiments and had the potential to be detected from field-collected leaves (Table 1). These findings suggest that herbivores leave genetic materials on their feeding marks, and that the eDNA can be detected by PCR amplification.

While eDNA analysis is typically used to detect aquatic macro-organisms (Ficetola et al., 2008; Fukumoto, Ushimaru, & Minamoto, 2015; Lacoursière-Roussel, Côté, Leclerc, & Bernatchez, 2016; Yamamoto et al., 2017), several recent studies have also reported the detection of terrestrial eDNA. For example, the eDNA of arthropods can be recovered from parts of terrestrial plants, such as flowers, leaf mines, and pitcher plant fluid (Bittleston et al., 2016; Derocles et al., 2015; Thomsen & Sigsgaard, 2019). Aquatic eDNA spreads widely in water, thereby being useful for inferring the presence or absence of organisms. In contrast, terrestrial eDNA is more stationary and, therefore, may be suitable for investigating organism behavior and interactions. Indeed, the results of the present study clearly demonstrate that the detection of eDNA from feeding marks can be used to identify interactions between plants and herbivorous insects. Thus, the detection of eDNA from terrestrial substrates is useful for elucidating both casual associations among organisms (e.g., which insects visit a specific flower; Thomsen & Sigsgaard, 2019) and more specific interactions (e.g., feeding, pollination).

In the present study, the amplification of herbivorous insect DNA was positively correlated with the rim length of feeding marks (Figure 2), which suggests that herbivore DNA was mainly recovered from the feeding marks. Similarly, mammalian eDNA has been attributed to the presence of saliva and inner mouth cells by two studies: One identified mammalian predators from salivary DNA left on the carcasses of endangered animal species (Imazato, Onuma, Nagamine, & Nakaya, 2012), and the other detected the DNA of Asiatic black bear left on damaged crops (Saito, Yamauchi, & Aoi, 2008). The present study just suggested the positive correlation between feeding mark size and PCR amplification rates in the experiment of *B. mori*. If the association pattern is generally observed in other herbivorous insects, the feeding rates of individual insect species could be quantified by our method although this idea should be assessed by further studies.

The detection of herbivore DNA from feeding marks can be an effective method for identifying plant–herbivore interactions and to possibly evolve in quantifying capacities. However, certain problems must be overcome when detecting herbivore eDNA from leaves collected from the field. First, it is possible that insect DNA could exist on both feeding marks and the leaf surface. In the present study, for example, *B. mori* DNA was amplified from a few leaves without feeding marks (Table 1), which suggested that DNA was amplified from either silk thread or small amounts of excrement. Indeed,

TABLE 1 The number of assessed leaves and leaves detected herbivorous insect DNA

Sample	Total leaves assessed	Leaves with successful amplification	
		Number of leaves	Mean amplification success among eight PCR replicates
Bombyx mori			
Leaf with feeding marks	13	9	4.3
Leaf without feeding marks	7	2	1.5
Lycaena phlaeas daimio			
Leaf with feeding marks	19	1	8

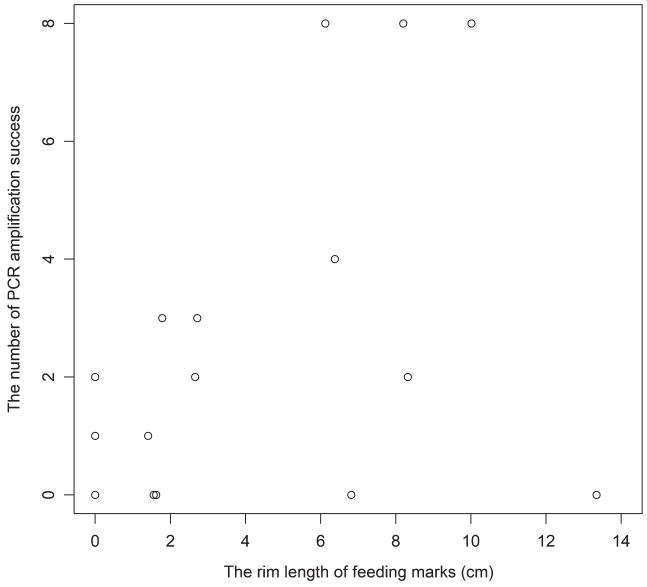

FIGURE 2 Scatter diagram illustrating the positive correlation between rim length of feeding marks and the number of successfully amplified PCR replicates among the eight PCR replicates. Spearman rank correlation coefficient was 0.510 (*p*-value <.05)

some studies have reported the recovery of eDNA from spider web thread (Blake, McKeown, Bushell, & Shaw, 2016; Xu, Yen, Bowman, & Turner, 2015), and in the present study, silkworms were observed to spin thread and defecate. Even though the leaves were checked visually to ensure that no contaminants remained, small amounts of excrement or thread could have been left behind. To reveal plant–herbivore interactions, it is better to extract eDNA from the feeding marks only; feeding marks should be isolated from other the parts of the leaves before eDNA extraction. In addition, it is also possible that amplifications from leaves without feeding marks were false positive because the rate of amplification from the leaves was 1.5 and that from negative control was 1. The negative control was one of the sampling blanks in the controlled feeding experiment; other blanks were not amplified. Therefore, positive detection from the negative control would likely be due to cross-sample contamination during eDNA sampling. It also could be due to contamination during PCR. Second, the eDNA left on feeding marks would likely degrade over time. Recent studies of eDNA detection have reported that the rate of eDNA detection is reduced by DNA degradation, which is caused by exposure to ultraviolet radiation, temperature, rainfall, pH, and microbial activity (Agetsuma-Yanagihara, Inoue, & Agetsuma, 2017; Barnes et al., 2014; Pilliod, Goldberg, Arkle, & Waits, 2014; Strickler, Fremier, & Goldberg, 2015). The present study demonstrated that the DNA of target species can be detected from feeding mark samples collected in the field, but positive amplification of the target DNA fragment was only achieved for one of the 19 samples, even though some of the feeding marks on the field samples seemed to be freshly grazed. It is likely that the unexpectedly low detection rate can be attributed to DNA degradation, and that this degradation could cause false negatives. Therefore, methods for detecting small amounts of DNA should be developed. For example, it might be more effective to collect leaves with fresh feeding marks by checking state of the marks and insects' excrement, and to then concentrate the extracted DNA solutions. It would also be important to attempt to remove PCR inhibitors in extracted DNA solutions; these inhibitors are often found in environmental samples. Methods for removing the inhibitors such as magnetic particles and DNA absorption columns should be assessed for their efficiency and their effects on eDNA concentration. To efficiently retrieve eDNA from feeding marks, cutting off leaves that were chewed on and extracting DNA from the leaf pieces might be also effective because some eDNA on feeding marks could not be completely washed off. Further, other methods with higher sensitivity would be more efficacious. For example, droplet digital PCR (ddPCR) was recently reported to be better suited for measuring eDNA than qPCR, especially at low DNA concentrations in water (Doi et al., 2015).

In conclusion, we suggested that the eDNA of herbivorous insects could be amplified from leaves with external foliar feeding marks and herbivores can be identified by sequencing the amplified DNA fragments. Improvements such as increasing the detection rate from degraded or small amounts of DNA will make this method more robust under diverse natural conditions. Furthermore, the combination of universal primers and next-generation sequencing will allow us to detect additional species and thus determine which insect species are herbivores of a specific plant. There are numerous leaves with feeding marks in the natural environment, and our method has the potential to use these to identify insect herbivores and quantify their feeding damage under natural conditions. This approach would be time-effective in comparison with traditional methods, such as direct observation in the field, and would thereby enable us to study large-scale interspecies interactions.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

A.K. and S.Y. designed the study; A.K. collected and analyzed the data; A.K., S.Y., and T.M. interpreted the data; A.K. wrote the initial manuscript; and S.Y. and T.M. revised the manuscript.

DATA AVAILABILITY STATEMENT

We have provided the data of primers and probe specificity test and sequences amplified from feeding marks in the Supporting Information.

ORCID

Aoi Kudoh https://orcid.org/0000-0002-2271-9193
Toshifumi Minamoto https://orcid.org/0000-0002-5379-1622
Satoshi Yamamoto https://orcid.org/0000-0003-0123-9112

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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