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# EXPLORATORY ENVIRONMENTAL DNA ANALYSIS FOR INVESTIGATING PLANT-FEEDING HABIT OF THE RED-EARED TURTLE USING THEIR FECES SAMPLES

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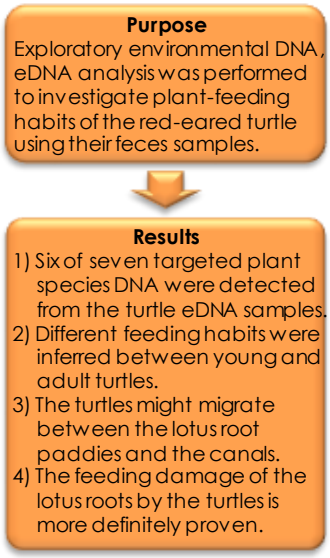
## Graphical abstract




## Abstract

Exploratory environmental DNA, eDNA analysis was performed to investigate plant-feeding habits of the red-eared turtle using their feces samples. This turtle species non-native to Japan appears to be causing feeding damage to lotus roots in several rural areas as a pest, although the reality is still unproven scientifically. The feces samples were collected from five turtles inhabiting agricultural canals surrounding lotus root paddies in Tokushima Prefecture where feeding damage has arisen. After eDNA extraction from the feces samples and polymerase chain reaction, PCR amplification, electrophoresis and sequencing analysis of the amplified PCR products were carried out to confirm whether chloroplast DNA fragments of seven targeted plant species including the lotus were detected from the eDNA samples. From the results, the DNA fragments of six plant species were detected from all eDNA samples, hence, this eDNA analysis appeared to be successful. It suggested that the number of the detected plant species differed between young and adult turtles. Different habitats of the detected plant species indicated that the turtles migrated between the lotus root paddies and the canals. The lotus DNA fragments were found in all turtles. Therefore, our eDNA analysis helps to more definitely prove the feeding damage of the lotus root by this turtle.

*Keywords*: Alien species, feeding damage, rural ecosystem



## 1.0 INTRODUCTION

The red-eared turtle, *Trachemys scripta elegans* (left in Figure 1) widely inhabits agricultural canals and ditches in rural areas around Japan. This turtle species, however, is non-native to Japan but native to the southern United States and northern Mexico. Many turtle individuals have been imported as pets since 1960's and were established in freshwater fields by pet releases [1]. We are concerned about the effect of this turtle on not only the habitats of other turtle species but also the Japanese rural ecosystem. Under this situation, the red-eared turtle has become a pest to an agricultural product. This turtle species appears to be causing feeding damage to lotus roots in Tokushima and Saga Prefectures [2-5], although the reality is still unproven scientifically. Especially, Naruto City in Tokushima Prefecture is famous as a major production area for lotus roots. Therefore, the turtle feeding damage to lotus roots has been the serious problem for farmers in the city

**Figure 1** The red-eared turtle (left) and an agricultural canal beside lotus root paddies where the turtles inhabit in Naruto City, Tokushima Prefecture (right)

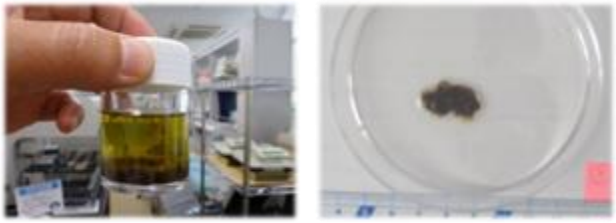


**Figure 2** A whole turtle feces sample preserved in 99.9% ethanol (left) and a part of a feces sample used for eDNA extraction (right)

[1, 5]. Investigations of feeding habits of this turtle have been required to clarify in detail the actual condition of the feeding damage at present.

In this study, exploratory environmental DNA, eDNA analysis was performed to investigate plant-feeding habits of the red-eared turtle using their feces samples. In fact, the turtle gastric contents and feces were examined by direct observation methods with a microscope in a few previous studies [6, 7]. It, however, is difficult to identify animal and plant at taxonomic species level from such digested samples. eDNA means generic DNA extracted from an environmental sample such as feces, water, soil, etc. eDNA contains cells derived from feeding and inhabiting organisms in a location. By detecting the targeted species DNA from eDNA samples, it is possible to validate the presence of the targeted species without direct observation and/or collection by traps, as the previous studies demonstrated [8-10].

We, fortunately, obtained feces samples of the red-eared turtle inhabiting agricultural canals in a lotus root paddy area in Naruto City (right in Figure 1). By applying the analysis of eDNA extracted from the feces samples, the kinds of plant species including the lotus that this turtle fed on were probatively examined to reveal its feeding habits.

## 2.0 MATERIALS AND METHODS

### 2.1 Feces Sample

Feces samples from 5 red-eared turtles were used in this study. These turtles were collected from agricultural canals surrounding lotus root paddies in Naruto City, from June to July 2014 (right in Figure 1). Based on classification of growth stages along with carapace length, CL [11], the turtles were divided into: two young females with 124 and 158 mm in CL, an adult male with 160 mm in CL and two adult females with 195 and 209 mm in CL. After feces samples were collected from the turtles in containers, the samples were immediately preserved in 99.9% ethanol (left in Figure 2) and stored at -30 °C until eDNA extraction.

### 2.2 Targeted Plant Species

Seven plant species were selected as targeted species of eDNA analysis (Table 1), based on the results of preliminary vegetation surveys. These plant species dominantly grew in and around the agricultural canals and lotus root paddies. The plant species are available as turtle feed. These plant

**Table 1** Common name, scientific name, habitat and chloroplast DNA fragment size amplified by polymerase chain reaction, PCR for seven targeted plant species (P1 to P7)

| No. | Common name | Scientific name | Habitat | DNA fragment size (bp [a]) |
|---|---|---|---|---|
| P1 | Lotus | *Nelumbo nucifera* | Lotus root paddy | 174 |
| P2 | Black medick | *Medicago lupulina* | Canal bank | 125 |
| P3 | Annual bluegrass | *Poa annua* | Canal bank | 167 |
| P4 | Undulate speedwell | *Veronica undulate* | Canal waterfront | 259 |
| P5 | Bitter cress | *Cardamine scutata* | Canal waterfront | 192 |
| P6 | Manchurian wild rice | *Zizania latifolia* | Canal water body | 335 |
| P7 | Reed grass | *Phragmites australis* | Canal water body | 202 |

[a] base pair

habitats were classified into four types: lotus root paddy, canal bank, canal waterfront and canal water body, with consideration for their distributions.

### 2.3 eDNA Analysis

#### 2.3.1 eDNA Extraction

The feces samples from 211 to 413 mg (right in Figure 2) were used for eDNA extraction with a QIAamp DNA Stool Mini Kit (Qiagen). The final DNA concentrations in 200 µL of eDNA samples were diluted by 0.106 to 0.754 ng/µL. The eDNA samples were stored at 4 °C until polymerase chain reaction, PCR amplification.

#### 2.3.2 Design of PCR Primer

PCR primers specific to the seven targeted plant species were designed by Rizo using International Nucleotide Sequence Database Collaboration, INSDC (http://www.insdc.org/). These PCR primers can amplify 125 to 335-base pairs, bp of chloroplast DNA fragments for the targeted species (Table 1). Before performing eDNA analysis, amplification of the expected size of DNA fragments was confirmed by the PCR primers using whole DNA extracted from body samples of the targeted plant species.

#### 2.3.3 PCR Amplification

Quadruplicate PCR amplifications for each eDNA sample were performed using the primer of the target plant species. Ultrapure water in four wells was also used as a negative PCR control template. Ten µL of a reaction mixture contained one µL of the eDNA sample and 250 nM of the targeted plant species primer was prepared according to the manufacturer's protocol of KAPA2G Robust PCR Kit (Kapa Biosystems). Thermal profiles on a C1000 thermal cycler (Bio-Rad) were as follows: initial denaturation at 95 °C for 3 min was followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 15 s.

#### 2.3.4 Confirmation of Positive PCR Product

Whether PCR products that mean DNA fragments amplified from eDNA samples by the PCR were positive for the targeted plant species was examined using the following procedure. First, DNA fragment sizes of PCR products appearing as DNA bands were measured using a microchip electrophoresis system (Shimadzu). Second, when the PCR product sizes were similar to the expected DNA size of the plant species (Table 1), DNA sequences of the PCR products were analyzed as follows. After purification using Agencourt AMPure XP kit (Beckman Coulter), the PCR products were directly sequenced on a 3130xl Genetic Analyzer (Applied Biosystems, ABI) using BigDye Terminator kit version 3.1 (ABI) and Agencourt CleanSEQ (Beckman Coulter). Third, when the obtained DNA sequences of the PCR products consisted with those of the targeted plant species deposited in INSDC using Basic Local Alignment Search Tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome), we determined that the PCR products were positive.

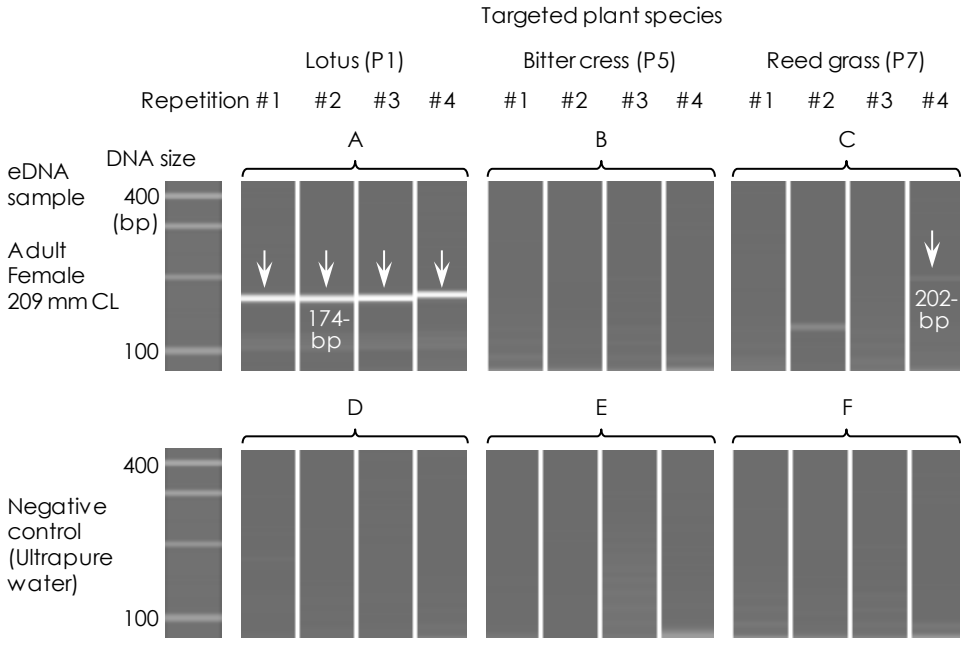



**Figure 3** Electrophoretic profiles of an eDNA and negative control samples examined for DNA fragments of three targeted plant species. The PCR amplification was performed in quadruplicate for the samples using the primer of the plant species (#1 to #4)

**Table 2** The number of turtles feeding on the targeted plant species for growth stage and sex based on the appearance of positive PCR products in the eDNA analysis

| No. turtles | Carapace length (mm) | Growth stage | Sex | Habitat | | | | | | | Total no. species |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Paddy | Canal bank | | Canal waterfront | | Canal water body | | |
| | | | | P1 [a] | P2 | P3 | P4 | P5 | P6 | P7 | |
| 2 | 124 and 158 | Young | Female | 2 | 1 | | | | 1 | | 3 |
| 1 | 160 | Adult | Male | 1 | 1 | 1 | | | 1 | 1 | 5 |
| 2 | 178 and 209 | Adult | Female | 2 | 2 | 1 | 1 | | 2 | 2 | 6 |
| | | **Total no. turtles** | | 5 | 4 | 2 | 1 | | 4 | 3 | 6 |

[a] P1 lotus, P2 black medick, P3 annual bluegrass, P4 undulate speedwell, P5 bitter cress, P6 Manchurian wild rice and P7 reed grass

## 3.0 RESULTS

### 3.1 Appearance of Positive PCR Product

Figure 3 shows examples of electrophoretic profiles of an eDNA samples examined for DNA fragments of three targeted plant species. DNA bands with arrows in Figure 3 indicate positive PCR products of the targeted plant species. Four, zero and one positive PCR products were found in the electrophoretic profiles (A, B and C in Figure 3, respectively); no DNA band appeared in negative controls including other targeted plant species (D, E and F in Figure 3). As applied in previous studies [8-10, 12, 13], we also presumed that the red-eared turtle fed on the targeted plant species when the positive PCR products were identified even if only one product.

### 3.2 Plant-feeding Habit Based on eDNA Analysis

Table 2 shows the number of turtles feeding on the targeted plant species for growth stage and sex, based on the appearance of positive PCR products in the eDNA analysis. Young females fed on a total of three targeted plant species; adult male and adult females were five and six targeted plant species in total, respectively. The targeted plant species on which young females and an adult male fed grew in three different habitats; those for adult females were in all habitats. The lotus (P1 in Table 2) was feed for all turtles. The black medick and the Manchurian wild rice (P2 and P6, respectively) were also common feed for young and adult turtles. No turtle fed on the bitter cress (P5).

## 4.0 DISCUSSION

### 4.1 Success of Detection for Plant Species DNA

Our eDNA analysis was successful in detecting DNA fragments of the targeted plant species contained in eDNA extracted from turtle feces samples, despite the exploratory analysis. In previous studies based on direct observation methods [6, 7], taxonomic levels of plants and animals identified from feces and gastric contents samples were only order and class. Compared to such identification levels, eDNA analysis that can identify organisms at species level appears to be superior to direct observation methods. Although it was not performed in this study, eDNA analysis can be applied to the investigation of turtle animal-feeding habits, when PRC primers of animal species are designed. The eDNA analysis methods should become a powerful tool to investigate details of feeding habits of various animal species, as the previous studies also described [8-10, 12-14].

### 4.2 Different Feeding Habit between Growth Stages

In this study, the targeted plant species were confirmed in eDNA samples of adult turtles more than those of young turtles (Table 2). This turtle species is basically an omnivore [15]. Young turtles, however, are mostly carnivores rather than herbivores; adult ones are herbivores more than carnivores. Different feeding habits between young and adult turtles were revealed in previous studies [16, 17]. Although only plant-feeding habits of the turtles were investigated in our eDNA analysis, this finding appears to reflect different feeding habits between turtle growth stages.

### 4.3 Migration between Lotus Root Paddy and Canal

Not only adult but also young turtles fed on several targeted plant species growing in different habitats (Table 2). Because turtles mainly inhabit agricultural canals, detected DNA fragments of the lotus, black medick and Manchurian wild rice growing in lotus root paddies, canal banks and canal water bodies, respectively suggests that the turtles migrated between the lotus root paddies and canals. And also our eDNA analysis showed that all turtles fed on the lotus. These findings help to more definitely prove the feeding damage of the lotus root by this turtle.

As future study subjects, investigation of migration timing, routes and manners should be expected to elucidate more details of the turtle's feeding behavior in lotus root paddies. Furthermore, based on obtained investigation results of the feeding behavior, development of devices such as barriers, traps, etc. can be designed to help protect against turtle invasion into the lotus root paddies.

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