A MASTER'S DISSERTATION

Assessment of environmental DNA for invasive freshwater turtle at Yeonhwaji pond in Jeju Island, South Korea

Department of Interdisciplinary Graduate Program in

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ABSTRACT

Environmental DNA (eDNA) could be a promising method for ecological surveys in the future, especially with endangered and rare species. eDNA method is a powerful tool for studying ecology, as it does not require direct taxonomic observation, thus allowing information about every organism present in the sample to be obtained, even at a cryptic level. Environmental DNA (eDNA) method has become an important tool for monitoring the latent emergence of species that can be difficult to find, such as invasive species in the early stages of their arrival and expansion, or after control or eradication efforts. Until recently, there have not been many studies employing the eDNA method for the detection of invasive alien species. I hereby conducted an assessment in Jeju Island, South Korea, for detection of invasive alien turtles using the eDNA method. The study was conducted at Yeonhwaji pond in the year 2021. An experiment to check the persistence of freshwater eDNA was examined in a lab setting. The field study was conducted under different weather conditions at a basking site and a non-basking site at different times of day. The result showed that the persistence of eDNA extracted from *Pseudemys peninsularis* was up to 21 days after removing the genetic source from the collected water sample. A total of 16, 4, and 0 samples were detected positively for *Trachemys scripta elegans*, *Mauremys sinensis* and *Pseudemys peninsularis* species respectively. Samples collected after rain and from non-basking sites showed a negative detection of eDNA. The results from this study demonstrated that eDNA can be applicable for species detection and surveying on Jeju Island, especially with freshwater or endangered species. However, due to the low positive detection rate, the eDNA method cannot replace the traditional methods, as expected. With its unique advantages, eDNA can, however, be combined with traditional methods for faster and more accurate surveys. Besides, this method may be employed to build distribution maps of species and detect endangered or rare species in the wild.

I. INTRODUCTION

1. General introduction

1.1. Invasive species

Globalization helps the wide and easy movement of people and goods brings many benefits, but it also leads to intentional and unintentional interference between separated ecosystems before (Perrings *et al*., 2010). An invasive species are species that newly introduced (mostly by human) to a habitat where they were not reported for their presence before. Invaders build their community in the new habitat and start their evolution (Simberloff, 2010). In some cases, many non-native species are beneficial in new environments, or are considered benign, others are considered detrimental and invasive (Andersen *et al*., 2004). The invasions in animals became one of the major reasons of conservation threat and leading to species extinctions (Simberloff, 2010). Alien species were spread from their home range via both purposeful and accidental reasons (Mooney *et al*., 2005). The invasion of alien species could cause plenty bad results such as evolutionary cycle substitution, lifecycle processes disturbance leading to the big losses of economy, and human health threat (Mooney *et al*., 2005). One of the significant reasons of global change is invasive species (Vitousek *et al*., 1996). Invasive species cause serious economic harm, an estimated billions of dollars are spent annually on these species (U.S. Congr. Off. Technol. Assess., 1993; Pimentel *et al*., 2000). Besides the economic impacts, biodiversity is also severely affected by invasive species. Many previous studies have reported the impact of exotic species on native species, community structure and ecosystems (Vitousek & Walker, 1989; Williamson, 1996; Wilcove *et al*., 1998; Parker *et al*., 1999; Sala *et al*., 2000; Mooney & Hobbs, 2000; Stein *et al*., 2000). Increasing numbers of exotic species in new

locations will severely affect local ecosystems (U.S. Congr. Off. Technol. Assess., 1993). Plenty permanent changes might be caused to native ecosystem due to invasive species by changing the structure and fecundity of native community, leading to extinction in some species (Andersen *et al*., 2004). In the contrast, invasive species open a big opportunity for biologists to test fundamental ecological processes and to protect the native habitats (Lodge, 1993; Sax *et al*., 2007; Richardson & Pyšek, 2008).

1.2. Reptile invasion

Recently, reptiles that are traded as pets have become popular in many parts of the world (Telecky, 2001). From there, they can become alien species because they easily enter any local ecosystems through escape or release by their owners. Native ecosystems may be severely affected or adversely impacted by exotic reptiles. The brown tree snake (*Boiga irregularis*) introduced to Guam after World War II is a well-known example of the destruction of native ecosystems by alien reptiles. The numbers of native birds, mammals and lizards are greatly reduced by this snake. Only 3 of the 13 bird species native to Guam are still in the wild (Rodda *et al*., 1997). The strongly invasive success of reptiles is due to the high introduction rate and the suitability of the alien species to the native living conditions such as climate, topography, soil… (Kolar & Lodge, 2001; Lockwood *et al*., 2005; Jeschke & Strayer, 2006; Blackburn *et al*., 2015; Mahoney *et al*., 2015).

Even native reptile communities are also disrupted by alien reptiles. The behavior and habitat use of a native species - *Anolis conspersus* – are changed by the invasion of non-native brown anoles (*Anolis sagrei*) on Grand Cayman Island (Losos *et al*., 1993). Although the fauna on the island is quite fragile and easily destroyed, the effects of invasions on native reptiles occur not only on the islands

but also on the mainland. In the United States, fire ant *Solenopsis invicta* had been first reported for their presence in 1918 (Willson, 1950). Later, many reports have documented this fire ant eating the eggs and young of native reptiles (Allen *et al*., 1997; Moulis, 1997). The extinction of the Texas horned lizard from its range was caused mainly by fire ants (Goin, 1992). Invasive species that affect native reptiles are not only animals but also plants. Exotic plants change the structure of the habitat, the structure of native plant, increase the frequency of fires, leading to serious impacts on the local turtle species (Steward, 1991; Lovich, 1995).

1.3. Freshwater turtles

Reptiles are vertebrate are cold-blooded species. Nowadays, 6500 species of reptiles have been found to exist in the world (Spilsbury, 2014). Freshwater turtles, marine turtles and tortoises belong to the order *Testudines* with 360 species confirmed in 14 families (Rhodin *et al*., 2018). Among 187 species are recognized Threatened in IUCN Red list, 127 species were ranked as Endangered and Critically Endangered (Rhodin *et al*., 2018). Terrapins, commonly known as freshwater turtles, are turtle species whose habitats are in freshwater or semi-aquatic freshwater such as ponds, lakes, rivers, and streams (Branch, 2012; Sayers & Kubiak, 2020). Currently, conservationists and biologists are interested in and conserving turtles because they are in the group of highly endangered species (Selman *et al*., 2013). Recently, a sharp decline in the number of turtles has been taking place all over the world (Van Dijk, 2000; Turtle Conservation Fund, 2002; Rhodin *et al*., 2015, Rhodin *et al*., 2018). Freshwater turtles are currently over-exploited in the Asian region for local markets and international trade. They are used for food, pet, or medicinal purposes (Cheung & Dudgeon, 2006). Human outdoor activities are one of the leading causes of turtles on the brink of extinction (Flather & Cordell, 1995;

Cordell *et al*., 2008). Besides, habitat loss or degradation, climate change and disease are also the main causes that threaten freshwater turtle population (Luiselli *et al*., 2021).

One of the most traded reptiles are the freshwater turtles. Before 1997, the red-eared turtle was the principally traded freshwater turtle. At one point, this species was accounted for 97 percent of all U.S. turtle exports (Telecky, 2001). However, in 1997, this species was banned from being imported into Europe due to the high risk of ecological damage (EU Regulation 338, 1997; EU Regulation 349, 2003). Since then, other species have been substituted in the freshwater turtle trade (Ficetola *et al*., 2012). Freshwater turtles are mainly traded in Europe as pets because they are easy to find and cheap (Van Wilgen *et al*., 2010). The introduced vertebrates such as birds, turtles, mammals have a huge possibility of establishing a community to be an invasive species (Jeschke & Strayer, 2005). The successful population establishment can be affected by climate suitability, long lived species, and enormously released (Ficetola *et al*., 2012; Leung *et al*., 2012). The alien turtle species could be harmful to the native turtle population via competing for food sources, habitat, or by hybridization (Rodder *et al*., 2009; Lambert *et al*., 2019).

Trachemys scripta elegans was reported for the first import to South Korea in the late 1970s. Millions of individuals of this species have been traded, mainly as pets. They are then released into the wild through people's activities and religious activities (Ministry of Environment, 2009). Following this, red-eared slider communities have formed despite government efforts to control by including the species in the list of invasive aliens with adverse environmental effects (NIE, 2015; Koo & Sung, 2019; Koo *et al*., 2020). After the red-eared slider was banned from being imported, other freshwater turtle species were imported instead (NIE, 2015). Over time, they formed different

communities next to the red-eared sliders and are constantly increasing in number of individuals (Mun *et al*., 2013; Koo *et al*., 2017).

2. Ecology and introduction of targeted species in Jeju Island.

2.1. *Trachemys scripta elegans*

Slider turtle of the genus *Trachemys* has 3 subspecies including *Trachemys scripta scripta, Trachemys scripta elegans and Trachemys scripta troostii* (Schoepff, 1792). Their natural distribution range is extremely vast along the Americas, across from the Great Lakes in the North to the Rio de la Plata in the South America (Ernst & Barbour, 1989; Legler & Vogt, 2013; Seidel & Ernst, 2017). *Trachemys scripta elegans* is a medium-sized freshwater turtle. Their carapace length can reach 350 mm with brown color (Scalera, 2006). The dorsal is oval, dark brown with yellow stripes while the plastron is yellow (Bringsøe, 2006). *T.s. elegans* is the most widely distributed among the 3 subspecies and considered as one of the 100 most invasive species of the world (Global Invasive Species Database, http://www.issg.org/database). They usually have a red or orange stripe behind their eyes (Ernst *et al*., 1994; Conant & Collins, 1998). In juveniles, many black spots appear on the plastron (Conant & Collins, 1998). This species is mainly carnivorous when young and becomes omnivorous when grows up. Their food is all available edible items surrounding such as small fishes, snails, aquatic plants (Newbery, 1984; Parmenter & Avery, 1990). In terms of habitat, this species are all aquatic species that prefer quiet waters with soft bottom such as rivers, ditches, swamps, lakes, and ponds (Bringsøe, 2006) and rarely leave the water, except for basking (Brown *et al*., 1995; Conant & Collins, 1998; Cox *et al*., 1998; Salzberg, 2000). The nesting season of this is from April to July while

mating season is from April to June (Bringsøe, 2006). In Republic of Korea, red-eared sliders were imported in 1970s for pet trade or religious purposes. This species has been banned from import to Republic of Korea since 2001 after approximating 6.5 million individuals were introduced here (Ramsay *et al*., 2007).

2.2. *Pseudemys peninsularis*

The genus *Pseudemys* comprises of seven species including *P. alabamensis, P. concinna, P. gougi, P. nelson, P. peninsularis, P. rebriventris, P. texana* (Zug *et al*., 2001). Among them, there are 4 species were report for their presence in Jeju Island, South Korea including *P. concinna, P. nelson, P. peninsularis, P. rubriventris* (Park, 2021). *Pseudemys peninsularis* – Peninsula Cooter turtle - is a medium to large freshwater turtle. The carapace length can be up to 400 mm in females and about 310 mm in males. The carapace color is normally dark brown and has the yellow to red stripes while the plastron is extremely big with light yellow to orange color. Head and neck generally have the yellow stripes (Seidel & Ernst, 1996). They are naturally distributed in North and Central of Americas (Seidel, 1994; Stuart, 1995). Cooter turtle (genus *Pseudemys*) generally are mainly found in freshwater areas such as rivers, lakes, or ponds. Sometimes, they can be observed in springs, swamps, or saltwater estuary. As a member of *Pseudemys* genus, Penisula Cooter turtle (*Pseudemys peninsularis*) normally can be seen while they are spending their time for basking (Rivera, 2008; Ernst & Lovich, 2009). Their main food is plant; hence, the habitat of these turtle has various types of aquatic plants or marine flora. However, they sometimes consume small aquatic species in the same habitat (Buhlmann & Vaughan, 1991; Ernst & Lovich, 2009). Breeding season of these species mainly focuses on summer when the weather is hot, mostly from late April to early July. From April

to October is their most active time during a year (Green & Pauley, 1987; Buhlmann & Vaughan, 1991; Jensen, 2008; Ernst & Lovich, 2009).

2.3. *Mauremys sinensis*

Unlike the above 2 turtle species, *Mauremys sinensis* is distributed in tropical regions of Asia such as Taiwan, China, Laos, and Vietnam (Chen & Lue, 1998; Rhodin *et al*., 2017). Chinese stripe-necked turtle can be adapted in various kinds of climate and habitat such as rivers, ponds, lakes, or reservoirs (Ernst & Barbour, 1989). This species was ranked as critically endangered in IUCN Red list and its population is decreasing over time (Li *et al*., 2021). *Mauremys sinensis* has high economic and nutritional value, so it is overexploited and one of the most traded species in the world (Shi *et al*., 2002; Kopecký *et al*., 2013; Masin *et al*., 2014). Besides, their natural habitat is also gradually being degraded due to various reasons (Chen & Lue, 2009). They are considered as an omnivorous species because their main food is small fish, snails, tadpoles, or aquatic plants (Bonin *et al*., 2006). *Mauremys sinensis* has characteristic yellow stripes on their heads. The carapace is dark while the plastron is pale yellow with black dots. The carapace length is about 20 cm (Jablonski *et al*., 2018). Chinese stripe-necked turtle's effects to ecosystems have not been well documented. The impact of this species on the habitat around them is also unknown. There are only few studies on them, but they mainly focused on their hybridization (Xia *et al*., 2011; Sancho *et al*., 2020) and their immune system (Liang *et al*., 2020; Khan *et al*., 2021).

3. Environmental DNA (eDNA)

To monitor populations and biomes, biologists need to determine the distribution and abundance of species. In the past, surveys have mainly used the visual encounter survey to determine the habitat of species. Ideally, all species in the survey area would be detected. However, some species may not be detected by traditional methods because of their low population numbers or their behavior. This has led to the potential to reduce the detection rate of some species and put rare species at risk of extinction (Wintle *et al*., 2012). This problem motivates scientists to look for new methods to supplement or replace traditional methods.

Metagenomics is related to collect the genome sequences from a living organism community in the same ecosystem. Metagenomics is the widest definition of DNA analysis from environment. Up to now, metagenomics was applied successfully for microorganisms (Hugenholtz & Tyson, 2008). Over the past decades, several studies have demonstrated that environmental DNA (eDNA) has been collected not only from microorganisms present in the environment but also from animals and plants. In the sediments, traces of the DNA of non-fossilized ancient plants and animals were also found. In 2003, DNA traces of extinct species on Earth such as woolly mammoths and moa birds were found in sediments in New Zealand and Siberia (Willerslev *et al*., 2003). Later, the scientists successfully collected environmental DNA of animals and plants in the surface soil (Hofreiter *et al*., 2003). Since then, environmental DNA techniques have been widely applied in many biodiversity studies in a variety of environments such as sediments, surface soils, ice, freshwater, and oceans (Haile *et al*., 2007; Ficetola *et al*., 2008; Gould *et al*., 2010; Foote *et al*., 2012; Yoccoz *et al*., 2012). Therefore, eDNA should be considered as an approach which can replaces or complements traditional methods.

Environmental DNA is a complex mixture DNA genomic collected in various types of environments and released by the species living in that environment and accumulated over time in different metabolic processes. This kind of DNA can be found in different sources such as skin flakes, feathers, feces, saliva (Taberlet & Bouvet, 1991; Olson *et al*., 2012; Davy *et al*., 2015; Hunter *et al*., 2015). Besides, environmental DNA techniques have also been successfully applied to monitor endangered animals (Piggott, 2016; Cardás *et al*., 2020). In China, researchers have been successfully employed eDNA method to early detect invasive golden mussel *Limnoperna fortunei* (Xia *et al*., 2018). In 2020, Kim Jeong-hui had detected fish diversity of 4 freshwater streams by applying eDNA method in Korea (Kim *et al*., 2020).

Environmental DNA surveys have been proven to have many advantages over traditional methods. First, it reduces the stress that a species might be experienced with the traditional methods. Previously, biologists often directly approached or captured species to study their distribution, numbers of individuals, or genetics. Secondly, it could reduce the risk of disease transmission between individuals or populations (Olson *et al*., 2012). During capture-and-release process, you might transport the infection from a species to a species in the mutual population or from the two different populations. The third advantage of eDNA is rapidity (Davy *et al*., 2015, Hunter *et al*., 2015). In nature, our target species are moving continuously, some of them are rare with small amount of individual or cryptic species. It requires us to spend more time for observing by traditional methods. This eDNA allows us to accelerate the study of the target species, thereby developing and planning conservation more effectively. Finally, scientists have given many examples of cost effectiveness of eDNA and proved that this technique is more economical than traditional methods. From the third

advantage, a person or a group of people who spend more time observing will be more expensive than a person applying eDNA technique in survey by reducing working-hour methods (Olson *et al*., 2012; Davy *et al*., 2015). The scientist calculated the cost of successful eDNA reading is cheaper than conventional methods (Davy *et al*., 2015).

4. Study purposes

Alien freshwater turtle species are suitable targets for an eDNA survey. Their escape from their owners or from religious activities leaves the distribution and numbers of these alien species uncontrolled in the wild. Knowing the distribution and status of freshwater turtle populations is extremely important. In the past, collecting data for these species was conducted by using VES (visual encounter surveys). However, there are plenty of challenges with this approach. At first, the locations of the study sites were normally far apart from each other. Next, alien freshwater turtle species are extremely difficult to observe because they spend most of their time swimming in the water or hiding under aquatic plants in their habitat. Lastly, their densities in new places are quite low as an introduced species. Hence, it takes many working hours to get reliable data, which can be a waste of time and money (Hunter *et al*., 2015).

Numerous studies have found that the persistence of eDNA in nature is from 14 to 60 days (Goldberg *et al*., 2015). Besides, plenty of publications assessed the effectiveness of the eDNA technique for target species, some of them concluding that environmental factors contribute to eDNA degradation. These studies hypothesized that higher temperatures and concentrations of dissolved

oxygen or more acidity could degrade environmental DNA (Corinaldesi *et al*., 2008; Poté *et al*., 2009; Barnes *et al*., 2014).

Previously, plenty of publications and studies have been conducted in Jeju Island about the distribution and ecology of alien freshwater turtle species. However, all of them were conducted by the traditional method – visual encounter surveys. Hence, my study has two objectives. First, I describe the development and testing of an eDNA assay that detects freshwater turtle species from a certain pond in Jeju. Secondly, I would like to assess the correlation of abiotic factors with eDNA detection. The results of this study will be the first step to propose a strategy about the use of the eDNA method in Jeju Island.

II. MATERIALS AND METHOD

1. Sample collection

1.1. Preliminary survey for housed laboratory individuals of *Pseudemys peninsularis*.

The target species living and operating in a certain area is the basis for the application of environmental DNA techniques. To detect the environmental DNA in a sample over time, 2 individuals of *Pseudemys peninsularis* were captured and placed temporarily in the Zoological laboratory to assess DNA degradation over time. The target species is still being reared in the laboratory for use in another study.

A transparent plastic container (58 centimeters x 38 centimeters x 27 centimeters) was used in this experiment to create a living environment for the target species. It was cleaned carefully with a washing solution and then filled with 4 - 5 liters of tap water. Two individuals of *Pseudemys peninsularis* were placed in that container for approximately 1 month at room temperature. They were supplied food every 4 days. After 1 month, the 2 individuals of *Pseudemys peninsularis* were removed from that container. The container, then, was kept in the same condition as before removal.

The same protocol as described below after the sample collection part was applied. Before each sample collection, water was mixed softly and carefully to diffuse particles inside the container. After water collection, there was no water added to the container. Water collected from the container before adding turtles was used as a blank solution. The positive control was *Pseudemys peninsularis* DNA extracted from tissue. For a negative control, extracted tissue DNA of *Trachemys scripta elegans* was used in this experiment. After removal, samples were taken at 1, 4, 7, 14, 21, 28 days and so on. For preventing the fault-negative results, samples would be collected the 2 continuous following days of

each negative result for reconfirmation. This experiment was replicated 3 times. All replications were treated similarly.

1.2. Study site

Jeju Island was established millions of years ago due to volcanic eruption and formed a special ecology (UNESCO, 2018). Jeju is an elliptical-shaped island with about 74 km long and 32 km wide. The total area of Jeju is 1849.2 square kilometers (Kang *et al*., 2018). Hallasan Mountain is the highest position located in the center of the island with an altitude of 1950m above sea level. The island is composed mainly of basaltic lava flows and small amounts of pyroclastic rocks and sediments (Lee, 1982; Park, 1994). On the island there are about 360 parasitic cones called "Oreum" (Kim & Son, 2018). There are 4 main ecosystems in Jeju such as alpine coniferous forest, temperate broadleaf forest, warm temperate evergreen lucidophyll forest, and temperate grass land. Jeju is also a special place when converges all 4 internationally important regions including: Jeju Volcanic Island and Lava Tubes World Heritage Site, Jeju Island Biosphere Reserve, Jeju Island UNESCO Global Geopark and Ramsar wetland sites (UNESCO, 2018).

Yeonhwaji - a pond in Haga-ri, Aewol-eup, Jeju-si - was chosen as a case study site for the first assessment of environmental DNA. It situated at latitude 33°27'17.47" N and longitude 126°20'50.86" E. It is located at an altitude of 69 m with total area up to 12,000 m2. It is surrounded by a rural area and next to a road with low density of human. There were 23 individuals of invasive turtle species had been reported in this pond. Among them, there were 7, 1, 0 individuals of *Trachemys scripta elegans*, *Mauremys sinensis* and *Pseudemys peninsularis* respectively reported for their presence in the study site (Park,2021).

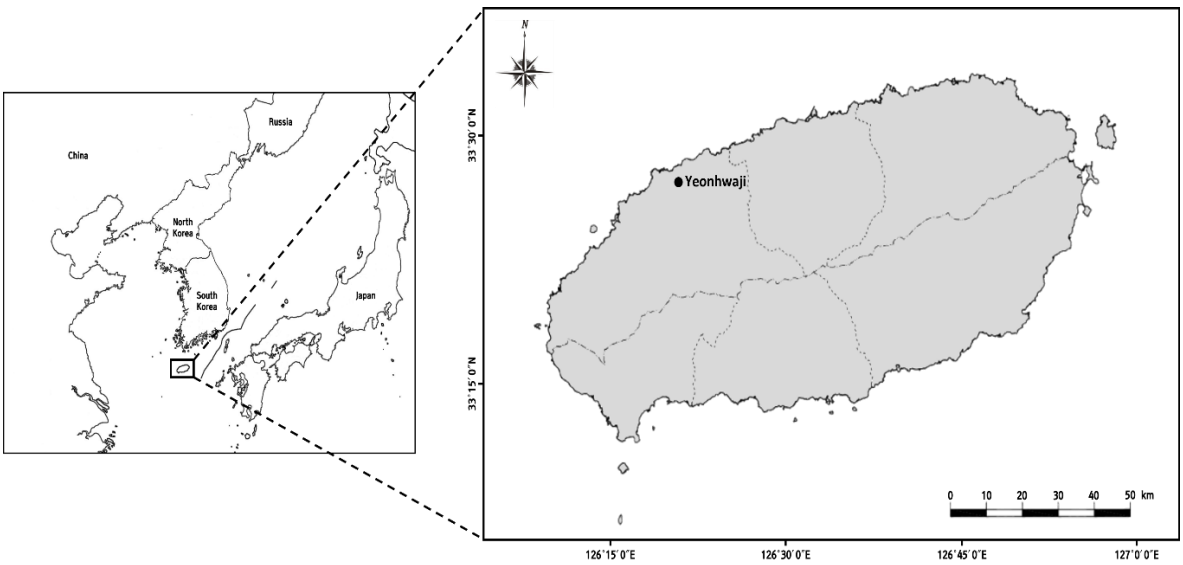


Figure 1. Location map of Yeonhwaji, Aewol-eup, Jeju Island, South Korea.


Figure 2. Surrounding habitat of study site - Yeonhwaji in Jeju Island, South Korea.

1.3. Visual observation

Prior to collecting water for each survey, a short visual encounter sampling was conducted to record the number of individuals or species observed by the naked eye. Biotic factors around and inside the study site were assessed. Biotic factors could be understood as biomass, life-history, metabolism or other species and food source in the same bio-ecosystem (Stewart, 2019). Here, the different animal species or plants would be reported as the biotic factors. They were identified by field guides and electronic devices. With unidentifiable species, they were captured or photographed and sent to experts for clarification.

1.4. Pond sampling

A couple of preliminary surveys were conducted to look for the species inside the pond and define the basking sites of freshwater turtles in this pond. This study was conducted during active times of freshwater turtles during the year in 2021. The surveys were mainly conducted at 3 different times in a day, such as early morning (around 7:00 a.m), at noon (around 12:00 p.m) and evening (around 6:00 – 7:00 p.m). Besides, the basic different weather conditions (sunny, cloudy and after rain) were also considered in collecting samples. Water was collected near the detected basking site in the study site with higher species abundance and a non-basking site with low species abundance. Basking sites are places which have exposure to the sun and are accessible to turtles for resting and observing the sunlight. A non-basking site is a place where exposure to sunlight is less and turtle activities are few. These places were identified on eDNA pre-surveys. DNA contamination is usually a big concern of the PCR protocol (Taberlet *et al*., 1996; Thomesen & Willerslev, 2015). Hence, before every field sampling, 50ml of distilled water was prepared in a cleaned container and treated as a negative control.

To minimize cross-contamination, a new set of equipment was used for each sampling day. With re-used items of equipment, they were carefully cleaned with a washing solution and placed under UV light for 10 minutes before their next use.

One liter of water sample was drawn from the study site into the plastic bottles. Prior to collecting water, these bottles were rinsed 2 to 3 times with pond water. Water was drawn at 20 to 30-cm depth from the surface of the ponds. There are 2 reasons for collecting water in this way. Firstly, the water at 30 cm depth would not be denatured by UV and sunlight or contaminated by rubbish like the surface water. Secondly, it is easy to collect water at this level. One more thing, water samples would not be contaminated by large sediments, such as dust from plants or soil at the bottom level. This would help in the filtration step. The water samples, then, were measured for abiotic factors by a specific meter and kept in a box with an ice bag inside. Next, they were immediately transferred to the laboratory. In the laboratory, water samples were immediately filtered or stored in a refrigerator for a maximum of 24 hours before filtering (Deiner *et al*., 2015).

1.5. Abiotic measurement

After water samples were collected and stored in 1-liter bottles they were immediately measured for abiotic factors. There were 6 abiotic factors that were measured to assess their effect on the detection of environmental DNA in water, such as: pH, temperature, dissolved oxygen, electronic conductivity, total dissolved solids and salinity. A dedicated meter (multi-function waterproof pH/Cond./TDS/Salt/DO meter supplied by AZ Instrument Corp. from Taiwan) was employed. This meter has 3 specific different sensors. Each sensor would be used separately. Before starting measuring, three probes were connected to the display. The meter, then, was turned on by pressing the

power button. The electrode was dipped into the water (it must be completely immersed into the water). Next, the water sample was gently stirred by the probe without creating any air bubbles. After approximately 1 minute, the reading stabilized. Hence, the data was ready to be recorded. The used probe was replaced by another one for other factors. The process was the same as the previous one. After using, the probes were rinsed with clean water. The pH sensor was stored by dipping in a neutral solution (pH=7.0) for calibration.

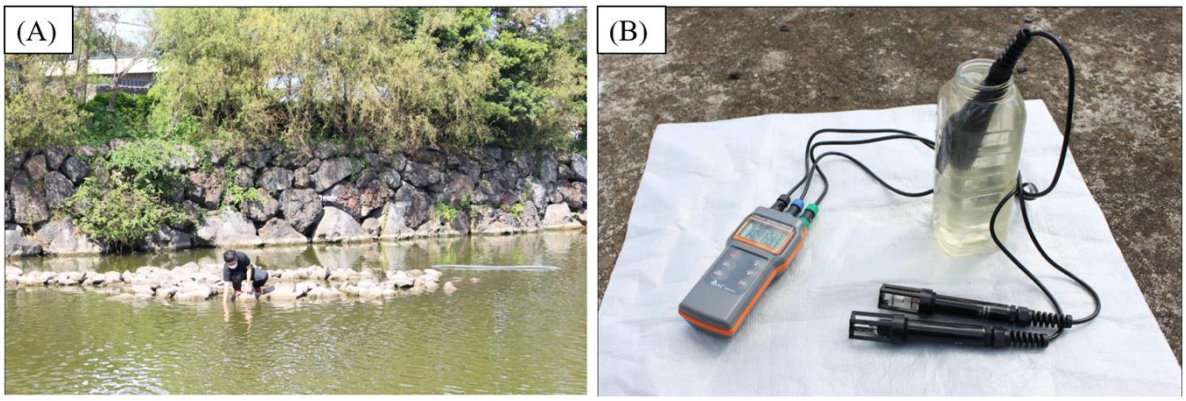


Figure 3. Field site work. (A) Collecting 1 liter of water sample at basking site; and (B) Setting up for measurement of abiotic factors in water samples collected.

2. Filtration.

Up to now, filtration is the easiest and primary method that scientists have employed to acquire environmental DNA (Renshaw *et al*., 2015). The water samples were always kept in a cooler to prevent DNA breakdown. During filtration, a new set of gloves was used for every sample. The filtration area was cleaned with 70% alcohol and covered with L25 air pocket wipers before starting every filtration process. The forceps and other equipment that were used in this process were washed, rinsed, and dried, then put under UV light for at least 10 minutes before using on the next samples.

Environmental samples drawn from the pond were still dirty with suspended sediments. Thus, prior to the filtration, the water was stored in the refrigerator for at least 5 minutes. This step was performed to settle down any large sediments to the bottom and prevent clogging the filter papers.

A GL45 Screw Connection 47 mm Vacuum Filter Holder set (supplied by Wheaton® in USA) was employed for using in this filtration step. It was attached to a peristaltic pump (Burkle® Hand Vacuum with Vacuum Gauge, supplied by Burkle® in Germany) following Hunter et al., (2015). A disposable Cellulose Acetate Membrane Filter with a 47 mm diameter and 0.45 μm (supplied by CHMLAB® in Spain) was used to separate genetic materials from water. After waiting for large sediments to settle down, a 1 liter of water sample was gently poured into the filter set with filter paper. The vacuum pump, then, was applied until all water samples were filtered. If the filter paper was clogged, another filter paper would be employed as an alternative and treated as another sample. A sterile pair of forceps was used to transfer the filtered paper into a 1.5 ml centrifugal tube. Next, the sample was immediately transferred to the extraction process. After every filtration, all the equipment was washed carefully and placed under UV light for 10 minutes. The filtration area was cleaned with 70% alcohol and made ready for the next experiment.

3. DNA Extraction

A DNeasy® Blood & Tissue kit (supplied from QIAGEN Group in Germany) was used in this step. The protocols for DNA extraction from water were followed according to the procedure provided by the supplier. All the equipment was disinfected under UV light before using. The

extraction area was wiped down with 70% ethanol prior to the start of each extraction. All chemical reagents were stored in a box at room temperature before using.

The filtered papers from the previous step were treated as tissue samples and the procedure for collecting tissues was followed. After filtration, all the cells collected in filtered papers are required to be broken down and lysed speedily in order to free nucleic acid. 180 µl of tissue lysis buffer ATL and 20 ml of proteinase K were added respectively to the 1.5 µl centrifuge tube containing the filtered paper from the filtration step. The tubes, then, were placed in a 56℃ incubator overnight to be completely lysed. During the incubation period, the tubes were taken out for vortexing a few times. After a 1-night incubation, the tubes were vortexed before adding 200 µl of lysis buffer to the tubes. Once again, the samples were vortexed and incubated at 56°C for 10 minutes. After incubation, 200 µl of ethanol was pipetted into the sample tubes and mixed by vortexing immediately. Next, the aqueous phase in these tubes was transferred to the spin column of a 2 ml collection tube. Then, the sample was centrifuged at 8000 rpm for 1 minute. The collection tube was discarded with flow-through, and the spin column was transferred to a new collection tube. 500 µl of AW1 and AW2 were added to the sample spin columns separately and respectively at 8000 rpm for 1 minute, then at 13500 rpm for 3 minutes. Collection tubes were removed after every washing solution. These steps are to remove all the contaminants, proteins, or salt. The final step in extraction is elution. For increasing DNA yield, the elution buffer AE was applied 2 times, separately. 100 µl was added to each sample in the spin column and stored for 1 minute at room temperature. Next, the samples were centrifuged for 1 minute at 9000 rpm. This step was repeated one more time without discarding the collection tube. The

collected solutions were then transferred into 1.5 ml centrifuge tubes, marked, and stored in a refrigerator at -20℃ until performing the PCR process.

4. Polymerase Chain Reaction (PCR) detection

Cytochrome b (*Cytb*) was chosen as the target gene to amplify eDNA samples. The species-specific primer pairs were collected for amplifying *Cytb* (Table 1) and checked for their specificity.

Table 1. Specific primers developed and used in this study to detect *T. s. elegans, M. sinensis and P. peninsularis* at Yeonhwaji, Jeju Island, South Korea.

Species	Primer	Sequence (5' → 3')
Trachemys scripta elegans	Forward primer	CCGAGACCTGTGGTTTGAAAAACC
	Reverse primer	GTCTTTGGTTTACAAGACCAATGA
Mauremys sinensis	Forward primer	CCACCGTTGTATTCAACTAC
	Reverse primer	CCGGGGATGAAGGTTTGGAGCC
Pseudemys peninsularis	Forward primer	CCGAGACCTGTGGTTTGAAAAACC
	Reverse primer	GTCTTCAGTCTTTGGTTTACAAGAC

Every extracted eDNA sample had to comply with the same conventional polymerase chain reaction procedures. All samples were run in a MiniAmp Plus Thermal Cycler (Thermo Fisher

Scientific, USA). The final volume for each PCR mixture was 20 µl, consisting of the following components: 3 µl of extracted eDNA sample, 1 µl of reverse primer, 1 µl of forward primer and 15 µl of distilled water. These 20 µl mixtures were added to the Maxime PCR Premix Kit (i-StarTaq). The thermo PCR cycling followed this protocol: an initial denaturation stage of 5 minutes at 95℃ followed by 40 cycles of 95℃ for 10 seconds of denaturation, annealing at 55℃ for 20 seconds, and an extension phase at 72℃ for 20 seconds. The final extension was at 72℃ for 5 minutes before storage at 10℃ until the next step. After approximately 1.5 hours of PCR processes, the amplified PCR products were run through the electrophoresis process for 25 minutes with a 1% agarose gel with Safe Shine Green stain and 0.5xTBE of buffer. The results were visualized under UV.

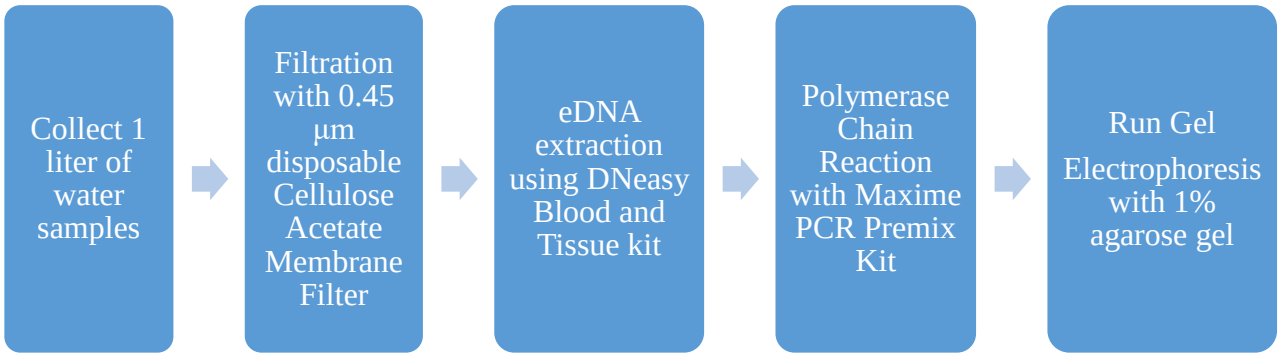


Figure 4. General procedure for eDNA detection of invasive freshwater turtle in this study.

5. Statistical Analysis

The SPSS version 16 system was employed to analyze the differences among variables and results of this study. The two-way ANOVA test was used to determine the average value of abiotic factors and which abiotic factors were statistically significant among different sites and weather conditions. In addition, the possibility of detection was analyzed by the Chi-squared test.

III. RESULTS

1. Preliminary survey for housed laboratory individuals of *Pseudemys peninsularis.*

Environmental DNA showed positive results in 5 of the 8 samples after taking the target species out of the containers. Positive detections were recorded in the samples collected on the 1st, 4th, 7th, 14th, and 21st days of the post-incubation period. The sample on the 28th day was negatively detected. Following that, samples collected on the continuous next days (29th and 30th days) were negative detections. The blank solution sample had no amplification of DNA. The negative control of *Trachemys scripta elegans* tissue sample had a negative detection. While the positive control from *Pseudemys peninsularis* tissue extraction successfully amplified the target gene as predicted (Figure 5).

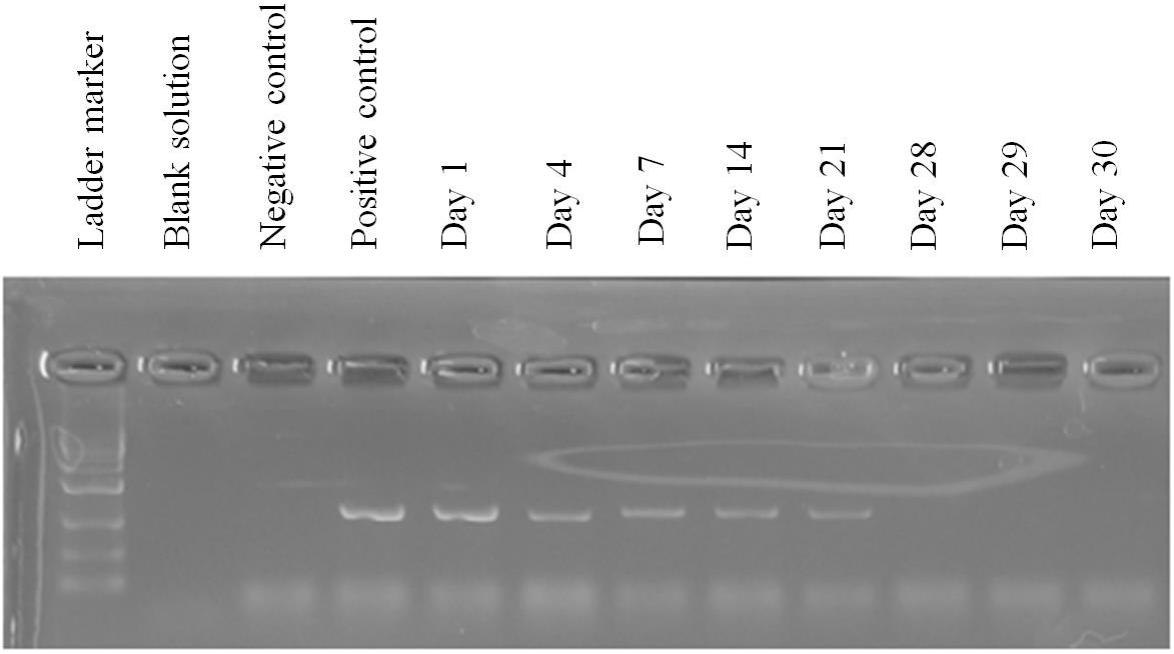


Figure 5. PCR product from *Pseudemys peninsularis* eDNA at lab setting over time after removing genetic sources 1, 4, 7, 14, 21, 28, 29 and 30 days. Product was run against 1Kb ladder. Negative control is *Trachemys scripta elegans* tissue extract.

2. Biotic factors

Under my observation and study, 21 species in total were recorded in the same ecosystem. For animals, 3 different species each of fishes, insects and microinveterbrates were reported, as well as *Rana nigromaculata*, which was the only amphibian that appeared in this study site. Another 7 freshwater turtle species shared the same habitat in this pond (Table 2).

Figure 6. Observed turtle species at basking site. A group of *Trachemys* genus at basking site.

Table 2. Surrounding biotic factors observed at study site.

Classification	Scientific name	Common name	Number	This study	Previous study
Fish	*Carassius auratus*	Goldfish	ND	o	
	Lepomis macrochirus	Bluegill fish	ND	o	
	Cyprinus carpio	Common carp fish	ND	o	
Amphibians	*Rana nigromaculata*	black-spotted pond frog	2	o	
Reptiles	*Rhabdophis tigrinus*	Japanese Keelback	1	o	
	Gloydius ussuriensis	Red-Tongued Viper Snake	1	o	
	Trachemys scripta scripta	Yellow-billed slider	1	o	o
	Pseudemys concinna	River cooter	3		o
	Pseudemys nelsoni	Florida red-billed cooter	1		o
	Pseudemys rubriventris	Northern Red-billed cooter	8	o	o
	Pelodiscus sinensis	Chinese Solfshell turtle	2	o	o
Insect	*Galerucella nipponensis Laboissiere*	Leaf bug	ND[1]	o	
	Crocothemis servilia	Scarlet skimmer	6	o	
	Orthetrum albistylum	White-tailed skimmer	5	o	
Macroinveterbrate	*Diplonychus esakii*	Giant water bug	ND	o	
	Gerris (Gerris) latiabdominis	Water strider	ND	o	
	Anisops ogasawarensis	Black swimmer bug	3	o	
Plant	*Salix babylonica*	Weeping Willow	ND	o	
	Trapa japonica Flerov	Water chestnut	ND	o	
	Iris pseudacorus	Yellow Iris	ND	o	
	Nelumbo nucifera	Sacred lotus	ND	o	

[1]ND is not determined

3. Abiotic factors

Overall, the two-way ANOVA test indicated that there was no significant difference among different sites or weather conditions concerning temperature, dissolved oxygen, electrical conductivity, total dissolved solids, and salinity. When comparing these abiotic factors at basking and non-basking sites or in sunny, cloudy and after rain conditions, all the P value were greater than 0.05, showing no difference among them. The average water temperature of the study site was 20.01℃ ± 3.62. Meanwhile, the mean of dissolved oxygen (DO), total dissolved solids (TDS) and electrical conductivity (EC) were 13.14 ppm ± 1.90; 129 ppm ± 3.48 and 257.77 µS/cm ± 7.29, respectively. Salinity was the most stable index among the 6 abiotic factors measured in this study. There was only 2 times that the salinity was measure at 0.14 ppt, while at all other times, it was steady at 0.13 ppt. In total, the mean of salinity was 0.1307 ppt ± 0.0025.

pH was the only abiotic factor that showed any significant difference among different weather conditions. The average pH of this pond is 8.11 ± 0.77. There was no significant difference between these 2 places ($P = 0.982 > 0.05$). However, it was significantly different among the weather conditions ($P = 0.00 > 0.05$). The average pH under sunny and cloudy conditions were 8.48 ± 0.46 and 8.40 ± 0.40, respectively, while the average pH after rain was 6.81 ± 0.16, and was dramatically lower than in the other 2 weather conditions.

Table 3. Two-way ANOVA statistical test for variations between basking site and non-basking site on abiotic variables.

Variables	**Site**		
	Mean square	**F**	**P**
Dissolved Oxygen	4.602	1.278	0.269
pH	9.277E-5	0.001	0.982
Temperature	33.232	2.588	0.121
Total Dissolved Solids	46.750	3.941	0.059
Electrical Conductivity	183.281	3.294	0.082
Salinity	5.182E-6	0.718	0.405

Table 4. Two-way ANOVA statistical test for variations among weather conditions on abiotic variables.

Variables	**Weather**		
	Mean square	**F**	**P**
Dissolved Oxygen	0.507	0.141	0.869
pH	6.251	35.916	0.000
Temperature	4.979	0.388	0.683
Total Dissolved Solids	3.167	0.267	0.768
Electrical Conductivity	5.660	0.102	0.904
Salinity	1.089E-6	0.151	0.861

4. Environmental DNA detection

The species-specific primers were designed using the *Cytb* gene sequence. When tested against other conspecific turtle species using a conventional PCR test, the non-target sequence was not amplified. All the negative controls did not show any amplified fragments.

Table 5. Target species identified by eDNA and visual encounter sampling method.

Sample number	Time of a day	eDNA			Direct observation		
		TSE	MS	PP	TSE	MS	PP
S1	Noon	o			o	o	
S2		o					
S3	Morning	o					
S4							
S5	Evening	o			o		
S6		o	o				
S7	Evening						
S8		o					
S9	Morning						
S10							
S11	Evening						
S12							
S13	Noon	o			o		
S14							
S15	Moring						
S16							

Table 5. continued

Sample number	Time of a day	eDNA			Direct observation		
		TSE	MS	PP	TSE	MS	PP
S17	Noon	o	o		o	o	
S18		o					
S19	Evening	o					
S20							
S21	Morning	o			o		
S22							
S23	Morning	o					
S24							
S25	Noon	o	o		o		
S26		o					
S27	Noon	o	o				
S28							
S29	Evening	o			o		
S30							

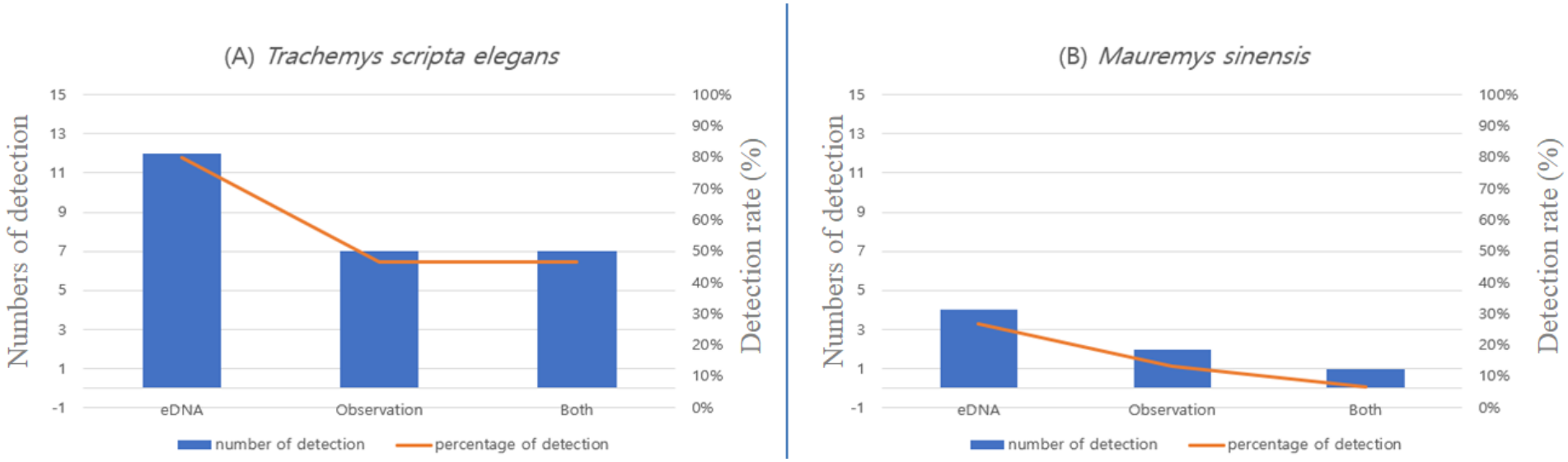


Figure 7. Target species identified by eDNA and visual encounter sampling method at Yeonhwaji, Jeju Island. (A) *Trachemys scripta elegans* and, (B) *Mauremys sinensis*.

For the water sample at the pond in Haga-ri, there was no positive detection of *Pseudemys peninsularis* in our study site. Meanwhile, the positive detection rate in *Trachemys scripta elegans* was the highest, with 53.3%, and negative detection was 46.7%. Lastly, *Mauremys sinensis* was detected positively, with only 13.3 %, while the negative detection rate was 6.5 times higher, compared to positive detection (Figure 8).

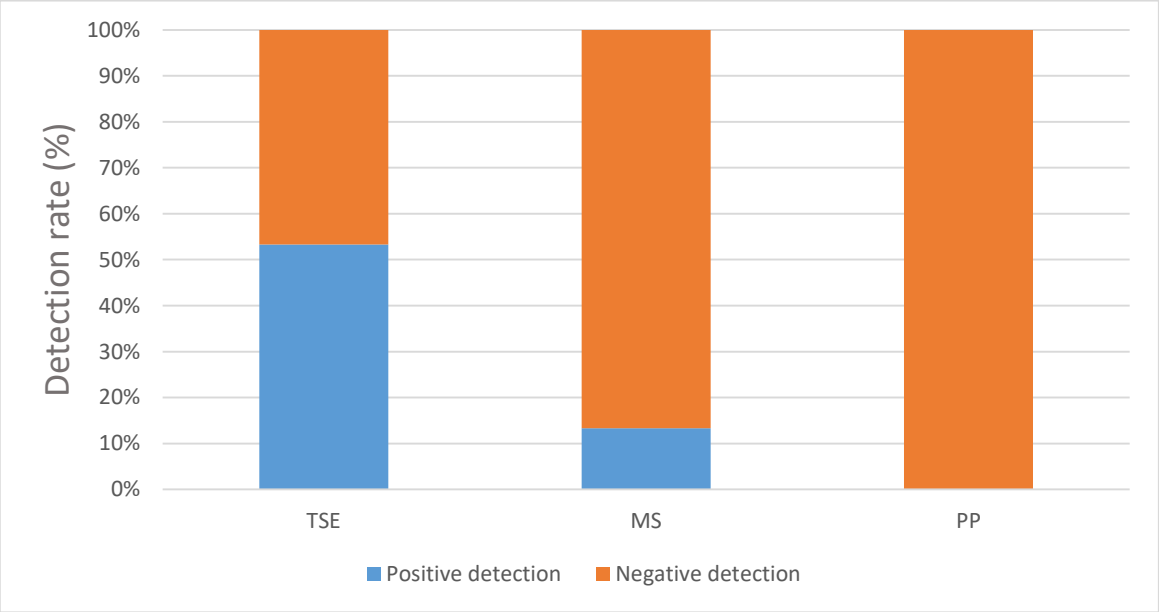


Figure 8. The ratio of positive and negative detection of 3 target species. TSE – *Trachemys scripta elegans*, MS – *Mauremys sinensis*, PP – *Pseudemys peninsularis*.

4.1. Detection of *Trachemys scripta elegans*

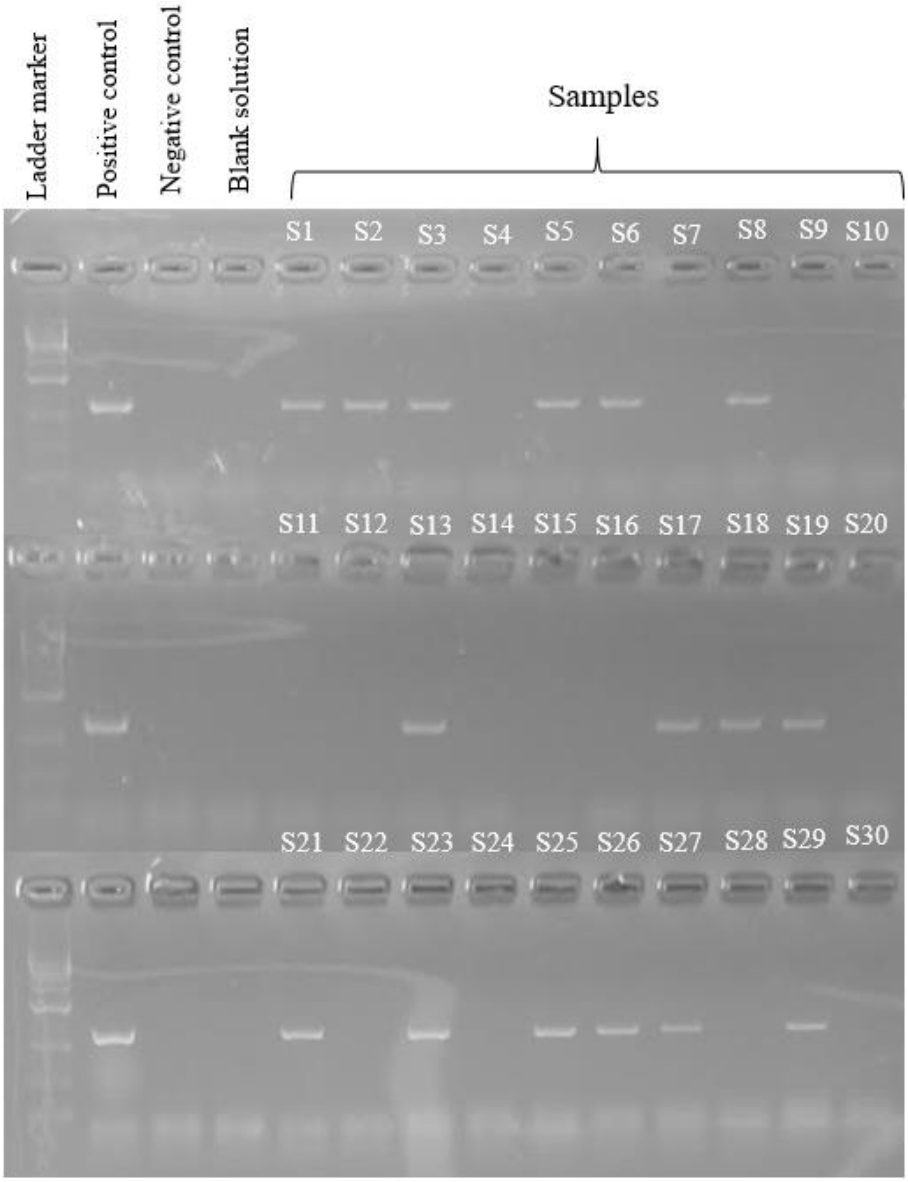
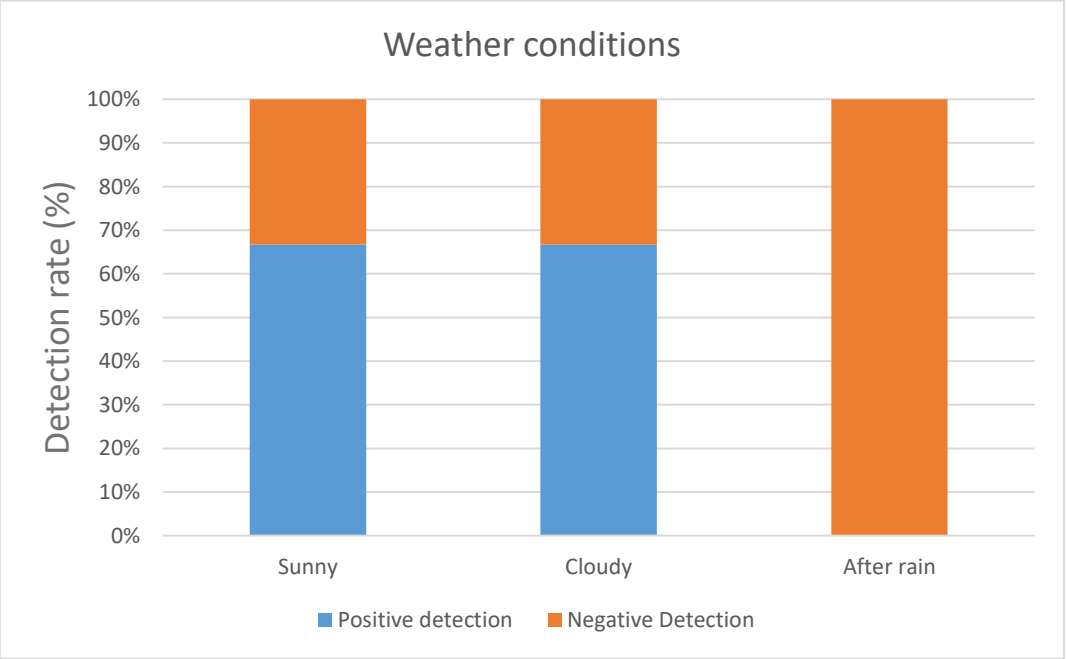


Figure 9. PCR results of *Trachemys scripta elegans* in a total of 30 samples. 16 water samples were successfully amplified using the eDNA of target species

Different sites and weathers conditions in this study significantly affected the positive detections of *Trachemys scripta elegans*.

In detail, the Chi-Square test indicated that the sites had a significant effect to the positive detection of *Trachemys scripta elegans* with $P = 0.011$ (<0.05). At the basking site, the positive detection rate was higher than negative detection, with 72.2% and 27.8%, respectively. Nevertheless, the negative detection rate was 3 times higher than positive detection at the non-basking site; it was 75% and 25%, respectively (Figure 10).

For different weather conditions, $P = 0.014$ (<0.05) showed the significant influence on the detection of target species. All the samples collected after rain did not show any positive detection. The negative detection was 100% in after rain samples. With sunny and cloudy conditions, the rates of positive and negative detection were similar to each other. The positive detection rate was double that of the negative detection, at 66.7% and 33.3%, respectively.



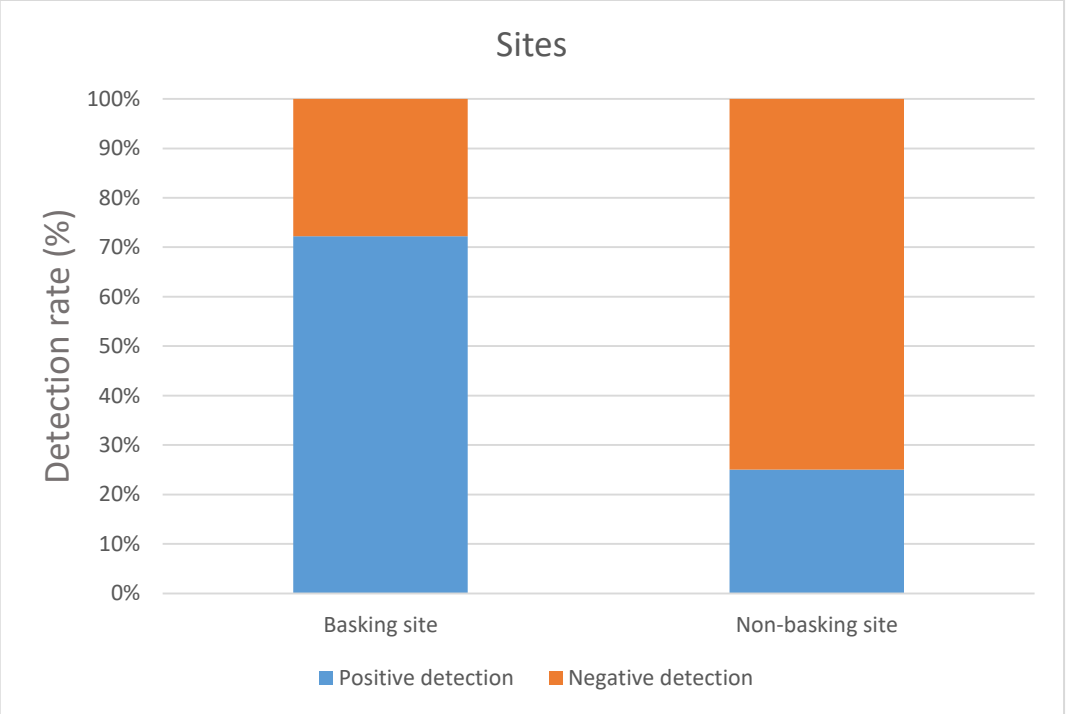


Figure 10. The detection rate of *Trachemys scripta elegans* among sites and weather conditions.

4.2. Detection of *Mauremys sinensis*

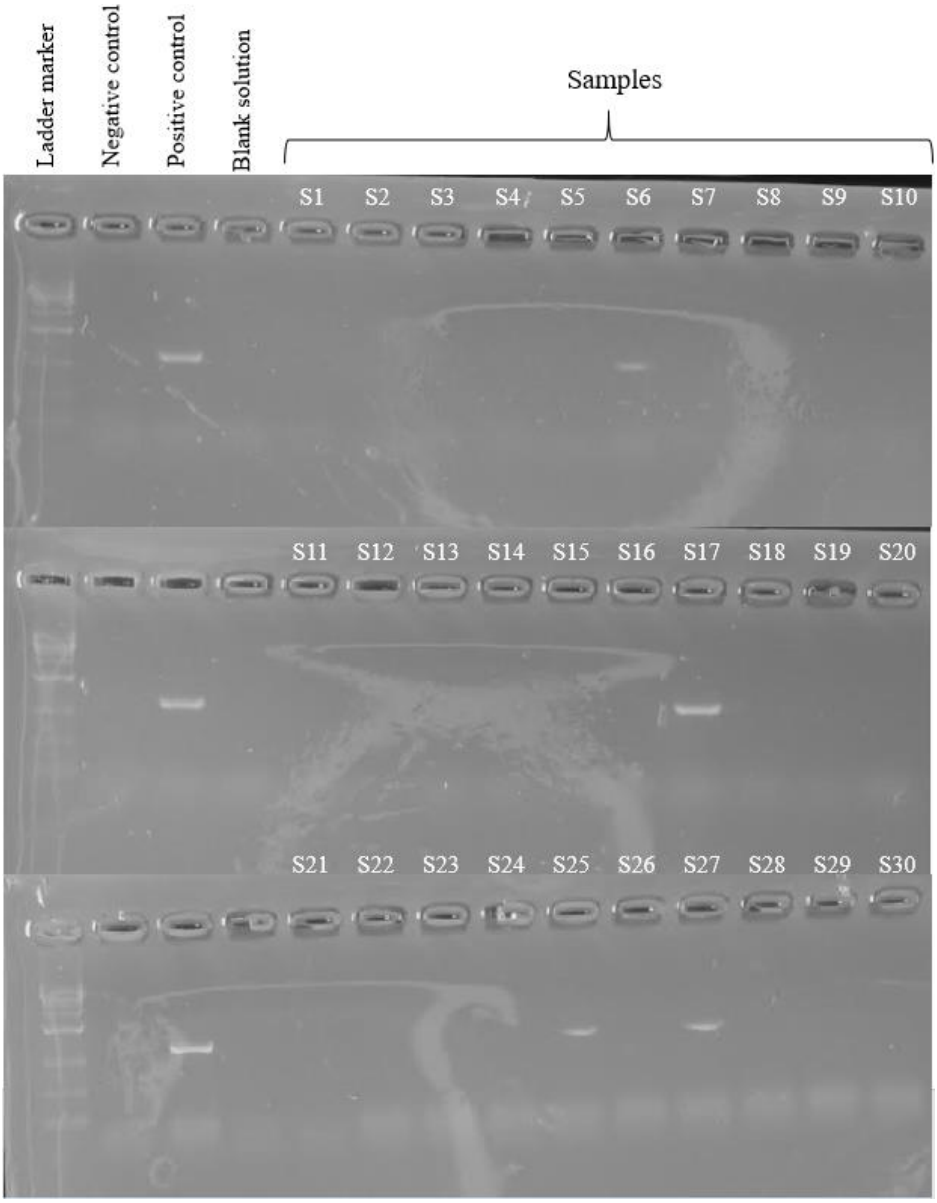


Figure 11. PCR result of *Mauremys sinensis* in a total of 30 samples. 4 water samples were successfully amplified using the eDNA of target species.

Chi-Square tests showed that all variables of sites and weather conditions were not significantly effective to the detection of *Mauremys sinensis*.

P = 0.079 (>0.05) indicated that sites were not related to the detection of the Chinese stripe-necked turtle. On the one hand, there wasn't any positive detection at the non-basking site, while on the other hand, the positive detection rate was 22.2%, and 77.8% with negative detection.

Besides, different weather conditions also showed no effect on the detection of target species through statistical analysis with P = 0.562 (>0.05). The positive results of environmental DNA were not detected after rain. The ratio of positive and negative detection under sunny conditions was similar to cloudy conditions. The negative detection rate in both of these weather conditions was 83.3%, while it was 16.7% of positive detection.

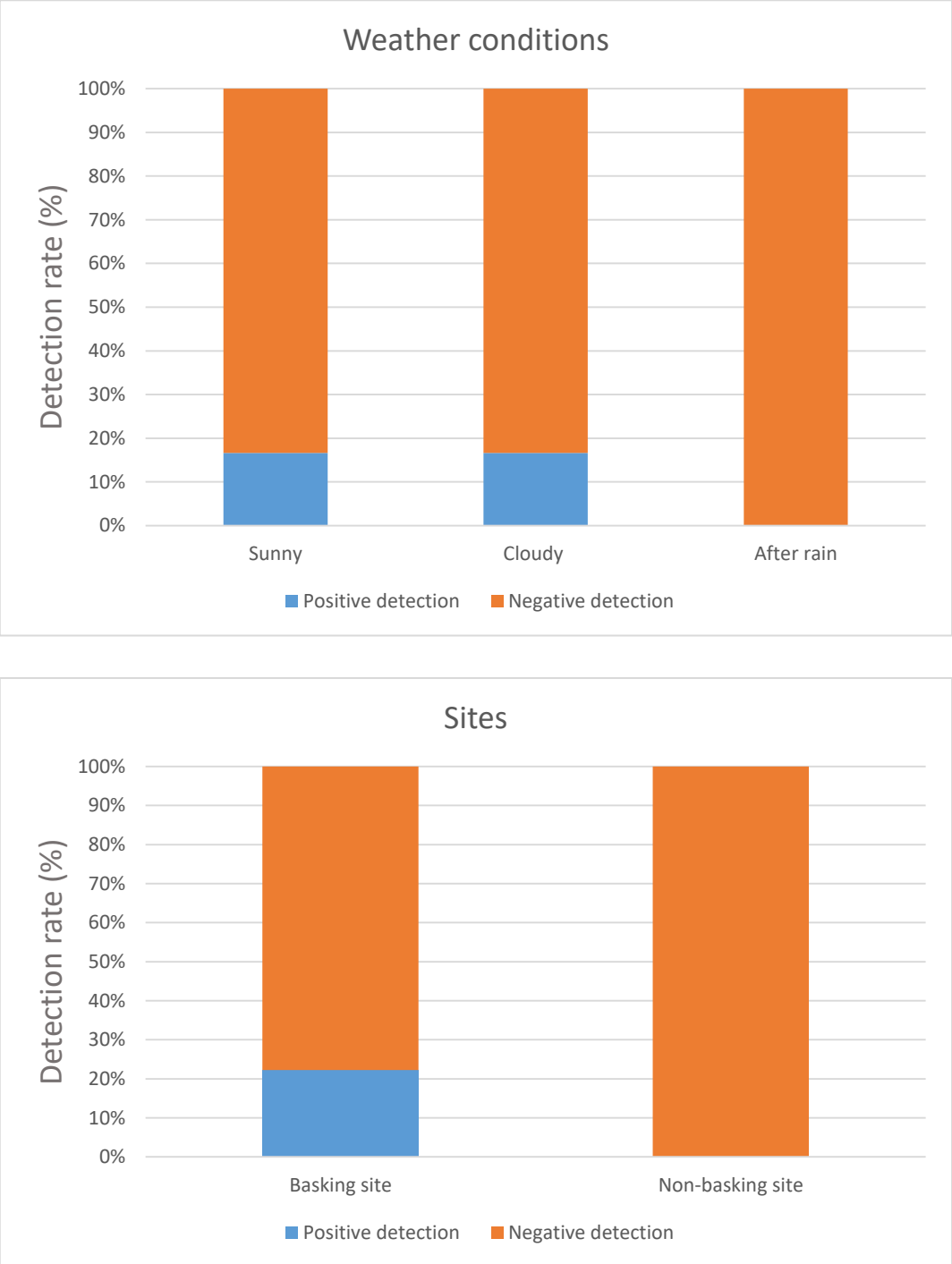


Figure 12. The detection rate of *Mauremys sinensis* among sites and weather conditions.

4.3. eDNA and visual encounter survey

I detected *Trachemys scripta elegans* by the visual encounter survey method for 7 out of 15 sampling days (46.6%). This species was detected positively be the eDNA method 12 times (80%), while they were detected by both eDNA and the conventional method 7 times (46.6%) (Figure 7).

For *Mauremys sinensis,* I detected their eDNA in 4 samples from different days among 15 sampling days (27%), while I could only observe them directly 2 out of 15 times (13%). Among those times, there was only 1 day in which they were detected by both eDNA and the conventional method (6.7%) (Figure 7).

IV. DISCUSSION

An environmental DNA assay for 3 alien freshwater turtle species was examined by this study. In order to achieve this assessment, 3 pairs of primers were employed using the *Cytb* gene to amplify specific sequences of the target species. Interestingly, eDNA samples were extracted from laboratory-caged water and natural pond water, known sites for the target turtle species. Besides, biotic factors around study sites were observed and abiotic factors of pond water were measured to assess their effect on the detection of eDNA. Lastly, eDNA samples of housed *Pseudemys peninsularis* were positively detected up to 21 days after removing the turtles from the container.

The primer pairs that were employed in this study are suitable for detecting 3 target species. Positive detection was found in 20 field site samples and in 5 lab samples. The specific primer pair for *Trachemys scripta elegans* used in this study amplified a product sequence of 1248 bp, while *Pseudemys peninsularis* aimed at a 1250 bp-length gene. Besides, the length of the PCR product amplified by *Mauremys sinensis* primer is 1281 bp.

1. Preliminary survey for housed laboratory individuals of *Pseudemys peninsularis.*

The results of *Pseudemys peninsularis* laboratory-housed samples indicated that eDNA could be found in water for 21 days after species removal. This result is between the 14 days to 60 days eDNA detection as examined by other studies (Santas *et al*., 2013; Goldberg *et al*., 2015). This experiment provides knowledge of using eDNA as a survey method for freshwater

turtles in the wild. Based on the percinacity of environmental DNA, the target species would not need to be immediately present at study sites to be detected so long as they had been present in that area within 21 days. Besides, the researchers would not need to recomfirm their presence on the sampling day. As we can see in Figure 5, the brightness of the band declined from the positive control from day 1 to day 21. This decrease of eDNA over time supports the value of using eDNA method in nature. The decline of eDNA appeared not only due to the original large number of target species, but also demonstrated the relative time interval for the species to have appeared in the study site (Barnes *et al*., 2014).

Moreover, those turtles defecated lots of fecal matter in the container. Pearson *et al*., (2015) demonstrated that fecal extracts were reliable for DNA tracing in reptiles. The relationship between concentration of DNA and time has been studied (Hunter *et al*., 2015).

The experiment of eDNA persistence and the decline of its concentration in this study was just conducted in a laboratory setting. It would be more reliable if a similar experiment could be conducted in a field site with natural interactions. Moreover, due to the lack of target species, only *Pseudemys peninsularis* was used for this observation. In the future, other alien turtle species should be assessed in order to collect the persistence of eDNA from other freshwater turtles.

2. Environmental DNA detection

There were 16, 4, and 0 positive detections out of 30 samples of *Trachemys scripta elegans*, *Mauremys sinensis*, and *Pseudemys peninsularis*, respectively. Interestingly, these results made sense with the previous studies which reported the number of individuals of our target species in Yeonhwaji. 7 individuals of the red-eared slider were documented, while only 1 specimen of the Chinese stripe-necked turtle was reported. The peninsula cotter has not been recorded for their presence in our study site.

2.1. Detection of *Trachemys scripta elegans*

Sixteen out of 30 samples showed positive detection (53.3%). The higher detection of the red-eared slider compared to the other 2 species in this study could be due to the higher density of individuals. The use of the eDNA method applied on the red-eared slider was well documented in previous studies. In 2019, positive detection in 20 out of 30 places (66.7%) confirmed the presence of *Trachemys scripta elegans* among 100 study sites (Kakuda *et al*., 2019). The sequence they targeted for designing primers was also a *Cytb* gene fragment. Interestingly, both studies showed low positive detection rates at places which were confirmed locations of the target species. This demonstrated that this method needs to be improved for more significant reliability over traditional visual methods, or further research could employ other target sequences for designing primers.

For sites, the positive detection was significantly different between the basking site and the non-basking site. The density of the target species was believed to be related to eDNA detection (Coulter *et al*., 2019). Because of this, the positive detection at the basking site was expected to be higher than the positive detection at the non-basking site. The result from this study proved this statement when the positive detection rate at the basking site was approximately 3 times higher than at the non-basking site. Normally, three to five individuals of red-eared slider gathered at the basking area. This produced a marked difference in the eDNA density, leading to a significantly higher positive detection rate at the basking site.

There was a significant difference of positive detection among the various weather conditions. The positive detection rates under cloudy and sunny conditions were similar to each other at 66.7%, while there were completely negative detections after heavy rain. While other abiotic factors did not show a significant difference, the acidity of the lake after rain was markedly lower than that of the other two weather conditions. Acidity is an abiotic factor that has been shown to affect eDNA (Seymour *et al*., 2018). The rain is slightly acidic and it decreased the pH of our study site. The pH level of rainwater is normally around 4.56 – 6.33, with an average of approximately 5.6 (Poghossian *et al*., 2001; Oduber *et al*., 2021). Strickler *et al*., (2015) proved that the low pH level showed a higher negative detection rate in eDNA due to degradation. The rain decreased the level of pH in our study site leading to the degradation of eDNA. Furthermore, those heavy rains also increased the water level of the

pond and diluted the density of eDNA. Hence, all samples collected after rain did not show any positive results.

2.2. Detection of *Mauremys sinensis*

The positive detection rate of *Mauremys sinensis* was 13.3% in this study. *Mauremys sinensis* detection employing eDNA has not been well documented yet. Baek et al., (2008) positively detected the Chinese stripe-necked turtle in 7 out of 15 study sites. This illustrated the extremely low detection rate of *Mauremys sinensis* in this study. More studies need to be conducted to increase the detection rate. Other protocols or chemicals could be tested for increasing the yield of eDNA in samples.

For sites, the difference of positive detection rate was not significant at the basking site and the non-basking site. Similarly, positive detection was not significantly different among the 3 stated weather conditions even though the pH was markedly lower after rain compared to the other two. A low detection rate could be the explanation for this. There was only 1 individual of *Mauremys sinensis* reported for its presence there. The density of eDNA from only 1 individual could not make a significant difference among variables in this study.

2.3. eDNA and visual encounter survey

In general, the eDNA detection rate was higher than in the visual encounter survey for both target species that I detected at the study site. Not surprisingly, the rate of detection by

each method was lower than for both methods combined. This result indicated the advantages of eDNA method compared to traditional methods. However, there was one time when I was only able to observe *Mauremys sinensis* visually while the eDNA result was negative. This illustrated that eDNA surveying is probably not useful as a stand-alone method, but rather as a way to assess study sites with the suspected presence of target species prior to more extensive, conventional methods.

3. Recommendations for furthur studies

Although this study positively detected alien turtle species by environmental DNA and proposed another method for surveying aquatic invasive species, improvements could be conducted and explored for more reliability. Replicates are necessary when we employ an eDNA method for surveying aquatic species for preventing fault-negative detection.

After collecting water samples at study sites, a chemical needs to be applied for preserving the eDNA. In this study, I simply kept bottles containing samples with ice bags. eDNA might be lost and start the denaturation process when we remove it from genetic sources and water. A few liters of water in bottles would be easily affected by outside conditions during transferring to a laboratory for filtration. Recently, benzalkonium chloride (BAC) was proven for its conservation of eDNA in freshwater species from denaturation (Takahara *et al*., 2020). The buffer for filtration and extraction should be considered to be

employed for quicker processing. eDNA in nature is mostly in small amounts or small fragments, hence, it needs to be preserved during filtering or extracting to reduce the denaturation.

The primer is also an issue that future studies need to notice. As I mentioned above, eDNA in nature exists in small pieces or fragments, so the primer pair should target small PCR products. In this study, I targeted the fragment over 1000bp, and it showed a low positive detection rate. The studies later should target small fragments or sequences for designing a primer, or choose another gene as a target. Nevertheless, the quantitative PCR test could be applied for quantification of DNA density. From that, we could estimate the abundance of the target species.

The eDNA degradation over time of one alien turtle species was demonstrated in this study. However, I just conducted it in a lab setting. It is essential to perform this experiment in the field site where eDNA and samples are affected by numerous environmental factors.

When employing eDNA methods for studying aquatic species, the survey should be conducted in sunny or cloudy weather and not on rainy days or the day after. It would therefore not be affected by the diffusion or degradation by pH decrease. The observation for freshwater turtle species should be surveyed in the day time or at noon when they are basking. The surveys conducted in the early morning or evening would be affected by the dark when there is a lack of light.

V. CONCLUSION

In spite of a low positive detection rate, the eDNA method can be applied in Jeju Island. The designed primer pairs isolating the fragments of the *Cytb* gene DNA sequence in this study can be employed to detect *Trachemys scripta elegans*, *Mauremys sinensis* and *Pseudemys peninsularis* eDNA extracts from freshwater ponds.

The eDNA method was expected to replace the traditional method – visual encounter sampling – in surveys in nature and the wild. However, the positive detection rate is low with 53.3% and 13.3% of *Trachemys scripta elegans* and *Mauremys sinensis* respectively at a site known to host target species. Because of this, the eDNA method is required to replicate results many times for each sample. Hence, eDNA cannot fully replace traditional methods in species surveys at this time in Jeju Island. It could be integrated with traditional methods for more reliable results in future surveys. It could also be used as a tool for quick assessment of suspected sites of target species before the main surveys. Twenty-one days is the longest time that eDNA of one of the target species could exist without a host in lab setting conditions. Research on other freshwater turtle species will be conducted to assess their eDNA persistence. With 16 positive detections of *Trachemys scripta elegans* compared to 4 and 0 positive detections of *Mauremys sinensis* and *Pseudemys peninsularis* at the same study site, species density showed a notable effect for eDNA detection (Yates *et al*., 2019). The positive

detection rate of target species at the basking and non-basking sites also supports the above statement.

In the future, eDNA studies can be applied to more quickly identify endangered and hard-to-see species in the wild, such as *Mauremys sinensis*. They can be employed to build distribution maps of target species, or build phylogenetic trees. With these advantages of the eDNA method, it is hopeful that many sites of target species can be rapidly identified, increasing the possibility of conserving the endangered species and protecting the native ecological environment. However, more eDNA evaluation studies need to be conducted to optimize this method.

When numerous species inhabit the same pond, like Yeonhwaji, it is hard to avoid collecting sediments. This may limit the samples, as eDNA from the sediment may overpower or diminish the small amount of eDNA present in the sample, though this inhibition was not examined in this study. Besides, the amount of eDNA loss was not counted during collecting water until extraction. A positive control or quantitative PCR could be applied in order to measure the eDNA loss from a sample collection to its extraction. The assessment of factors affecting eDNA detection was not fully evaluated in this study. Numerous other factors were believed to contribute to degrade the eDNA collected, such as DNA loss, biomass, metabolism, light, … More studies will be required to fully understand which natural factors could lead to negative detection of eDNA on Jeju Island.

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