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Prey Identification of the Little Tern, *Sternula albifrons* (Pallas, 1764), by Applying DNA Barcoding to Fecal Materials

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Abstract: This study describes the prey DNA fragments found in the feces of a migratory bird species, the little tern, *Sternula albifrons* (Pallas, 1764), based on a DNA barcoding approach. This species is found in Nakdong Estuary, South Korea, and is a species designated as 'Least Concern' (IUCN Red List). Prey identification is a central issue of population conservation, and we applied DNA barcoding (using cytochrome oxidase I; COI) to fecal materials from little tern individuals. We successfully identified prey consumed by little tern individuals. All prey items comprised one phylum including three classes, six orders, and eight families based on a robust dual certification scheme (combined analysis of BLASTn searches and phylogenetic tree construction). Even though the success of identification was largely dependent on the degree of completion of the database in the genebank or BOLD systems, an increased resolution of prey identification to species level is important in predator–prey research. The current study used a small number of fecal samples to evaluate the applicability of the COI barcoding region to avifaunal feces, and more fecal samples are expected to convey increased information that can be used to infer the range of the prey species of little terns.

Keywords: migratory bird; feces; dual certification; resolution of prey identification; COI barcoding region

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1. Introduction

Prey item identification is fundamental for understanding ecosystem structure and function [1]. The investigation of prey consumption patterns helps to evaluate the integrity or health of food webs and facilitates the development of ecosystem approaches. Currently, some techniques, such as stable isotope analysis, facilitate in-depth approaches to discovering functional interactions [2,3]; however, analyses should be based on the exact identification of the structural linkage between predators and prey. Recent advances in molecular biological techniques have supported this idea [4,5] and are recognized as powerful alternatives to conventional identification methods such as direct observation. DNA barcoding is a proven approach to significantly enhance the understanding of food web structure and prevents misidentification primarily based on the completeness of the search database, which leads to an improved identification resolution (i.e., at the species level) [1,6,7]. This method has popularly been applied to various ecosystems, and successful applications promote a more comprehensive understanding of food web dynamics [8].

Birds are a top predator in food webs of aquatic/wetland habitats, and investigation of their prey facilitates the determination of the structure and function of the underlying food web. The identification of bird prey items is thus important for identifying characteristics of food web dynamics in their habitats. However, the identification of bird prey consumption patterns often suffers from an insufficient resolution [9]. Generally, the observation of prey

consumption, which is made from a distance, is the classic, and still the most popular, way of inferring a predator's prey [10]. Observations are sometimes restricted if the bird population is endangered and is intensively conserved. Furthermore, observation distance often hinders exact prey identification, and specialized experts are often needed to conduct these observations. Considering the significance of birds in food web dynamics, a high resolution of prey identification (i.e., to the species or genus level) is crucial; consequently, more efficient prey-identification methods are necessary.

In this study, we propose an improved process of prey species identification based on the application of DNA barcoding to fecal materials of a migratory bird species. Because of the nature of fecal materials (i.e., denaturation or degradation), prey species identification from this type of media should be more carefully accomplished. We suggest adopting dual certification of prey species identification by means of sequence identity and phylogenetic comparison, which has been emphasized by Jo et al. [1]. To evaluate this process, we collected fecal materials of a migratory avian species, little tern, *Sternula albifrons* (Pallas, 1764).

Little terns, which are frequently observed in southeastern South Korea and distributed worldwide, are known to mainly feed on fish and invertebrates, which they swallow whole [11]. The distribution range extends to Europe, Africa, Asia, Australia, and even Hawaii [12,13], and the global population is relatively large (ca 190,000–410,000 individuals) [14]. However, the overall abundance of the species is decreasing, although it is currently still designated as a species of 'Least Concern' on the IUCN Red List. Prey identification of this species may help to improve our understanding of the species' predation characteristics and develop management strategies to conserve the species in their natural habitat.

We obtained fecal samples of this species from Nakdong Estuary, which is a breeding habitat of the little tern [11,15,16], and PCR amplification followed by cloning was applied to extract DNA from fecal samples. The following issues were evaluated in this study: (1) The potential of using standard cytochrome oxidase I (COI) for prey identification from fecal materials, (2) the identification of little tern prey species in a dual certification scheme, and (3) the determination of the number of clone samples needed to ensure the stability of species identification.

2. Materials and Methods

2.1. Study Site Description

Nakdong Estuary is located in southeast South Korea (35°05′ N, 128°55′ E; Figure 1) and provides important habitat for migratory birds in the Australasian Flyway. A variety of avian species utilize the estuarine area as their breeding or stopover site [17,18]. Annually, more than 200,000 individuals belonging to approximately 300 species of birds are found in the estuary, and the dominant species is the Mallard, *Anas platyrhynchos* (Linnaeus, 1758). The value of the estuary can be determined by the distribution of endangered species that are designated on the IUCN Red List [13].

The target species of this study, the little tern, typically arrives at the estuarine area in mid-April to breed and usually departs between July and August [16]. Their primary habitat is a small islet located in the southeastern part of the estuary [11,15]. Before 2000, this islet was frequently sunken by tidal movement, which hindered the species' ability to breed. This islet has been well exposed since 2000, and is almost always exposed [19], except during the rainy season when monsoon rainfall floods the Nakdong River. Typically, 2000 to 6000 individuals visit the islet in April and create nests in sufficiently elevated locations that have little vegetation (e.g., dry sand) [15]. The feeding behavior of the little tern has previously been investigated in this islet based on the observation method. According to Hong et al. [17] and Jang [11], the species is believed to consume small fish or shrimp. However, the results of these investigations were unable to provide a sufficient resolution of prey identification.

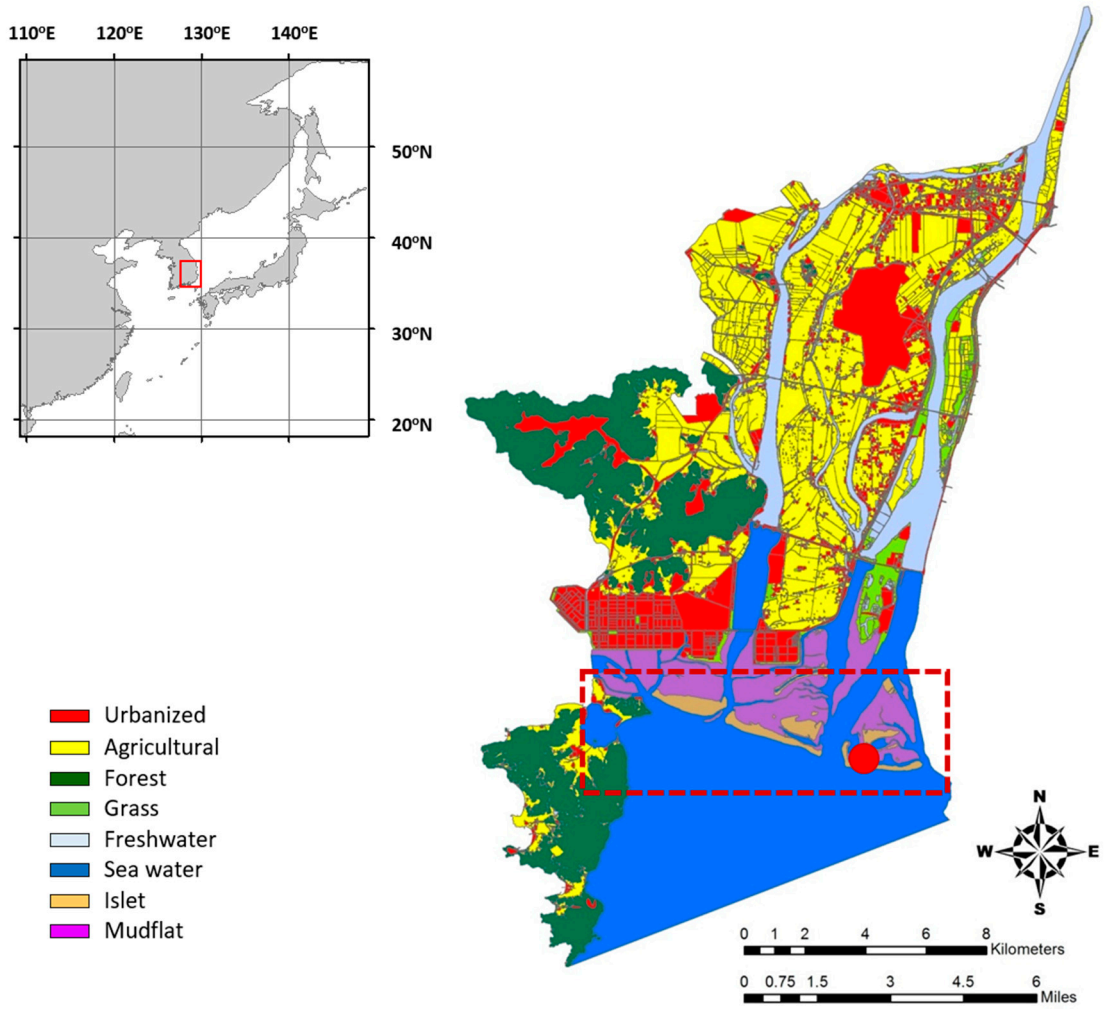

Figure 1. Map of study site (red circle: Sampling point in Doyodeung Island).

2.2. Sample Collection

The collection of little tern feces was conducted in May 2013, which is the time of year when the species typically attempts to nest and lay eggs. To ensure that the feces belonged to little tern individuals, we first explored the location of the nests in the little tern colony. Before sampling, we confirmed that the birds were far away from their nests (at least 200–300 m away from the colony) in order to not disturb their egg brooding behavior. When the birds departed from their nests, we carefully approached their nests and obtained their feces. Relatively fresh samples (excreted within 2 h) were obtained. Feces that seemed to be excreted earlier (very dry and mostly covered by sand) were excluded from sampling to avoid contamination with foreign DNA and excessive degradation. Feces were immediately stored in 95% ethyl alcohol after collection to minimize further contamination. Because of the presence of another bird species during this period of time (the Kentish Plover, *Charadrius alexandrines*), sample collection was carefully implemented by avifauna experts.

2.3. DNA Extraction, PCR Amplification, and Cloning

Ethanol was completely volatilized from the samples prior to DNA extraction. The samples were then frozen with liquid nitrogen and homogenized. Genomic DNA from each of the fecal samples was isolated using the Qiagen DNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's manuals. Between the homogenization of each fecal material sample, the mortar and pestle were cleaned using de-ionized water, and

any remaining material was burnt off using methanol to prevent cross-contamination of samples.

PCR amplification was performed using the AccuPower Hot Start PCR PreMix (Bioneer, Seoul, Korea) with genomic DNA and primers in a final volume of 20 μL. The COI region was amplified with LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTG-ACCAAAAAATCA-3′) [20]. The PCR consisted of 1 cycle of initial denaturation (94 °C, 10 min) and 40 cycles of denaturation (94 °C, 1 min), annealing (50 °C, 1.5 min), extension (72 °C, 1 min), and 1 cycle of final extension (72 °C, 10 min) by a Mastercycler (Eppendorf, Hamburg, Germany). PCR products were separated on 1.5% agarose gels. After purification using a Labopass Gel Extraction Kit (Cosmogenetech, Seoul, Korea), cloning was carried out using the pGEM-T Easy Vector (Promega, Madison, WI, USA).

Cloned plasmid DNA was cleaned up according to the alkaline-lysis method by a Labopass Plasmid Miniprep Kit (Cosmogenetech, Seoul, Korea). A plasmid DNA was isolated individually and then digested using the restriction enzyme EcoRI to confirm insertion. Thirty to thirty-seven positive clones for each sample were analyzed to produce species-specific sequences with SP6 primers using an automated 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), except for 2 samples (C-6 and C-9), which were analyzed for whole clones of each sample.

2.4. DNA Sequence Analysis and Statistics

ClustalW 2.0 [21] were used for sequencing alignment. To find the best hits, a BLASTn [22] search was performed. Ten sequences of the top hits from Genbank [23] and BOLD systems [24], as well as 3 or 4 outgroups from the nearest families, were downloaded. The degree of similarity between obtained sequences was assessed using the neighbor joining (NJ) algorithm [25] as implemented in MEGA-X [26]. The degree of information redundancy in fragments was assessed by bootstrap resampling of 1000 pseudoreplicates [27].

We used 2 criteria according to the methods described by Jo et al. [1] to establish accurate species identification: (1) The identification of an operational taxonomic unit (OTU) with ≥98% identity with a known species and a 2% difference between an OTU and a known species, which may be caused by intra-specific variation or PCR and sequencing errors [28,29]; and (2) that the phylogenetic tree constructed returned reasonable clustering of the sequences of the OTU and known species.

After OTU identification, the determination of sufficient sample size and clone numbers was implemented. We used a statistical shell PAST [30] to develop rarefaction curves for fecal samples using raw data. First, fecal samples were grouped based on the number of identified OTUs. Then, the number of clones was compared with the number of OTUs, and rarefaction curves were developed based on the data. The associated results indicate the numbers of clones and samples that are recommended for identifying the given number of OTUs.

3. Results

3.1. Identification of Prey Species by DNA Barcoding

Cloning analysis for DNA barcoding provided a list of consumed prey items at a reasonably high resolution from 11 fecal samples of the little tern (Table 1). All samples were successfully amplified by PCR. We sequenced 374 clones of prey items and obtained robust 658-bp sequence data; the clones contained DNA from 10 prey items. Among these, 9 out of 10 of the prey items were clearly identified at the species level. The identification accuracy of the remaining prey item could only be identified at the genus level.

Table 1. List of prey items identified by COI barcoding (QC: Query cover, ID: Identity, IL: Identification level, Sp: Species, Ge: Genus).

Phylum	Class (Subclass)	Order	Family	Genus + Species	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	Sum	Genebank	QC	ID (%)	IL
Chordata	Actinopterygii	Clupeiformes	Engraulidae	*Engraulis japonicus*	1											1	AB040676.1	99	99	Sp
			Clupeidae	*Clupea pallasii*	11	23			35	9	9	27	25	1		140	JF693636.1	100	100	Sp
		Cypriniformes	Cyprinidae	*Hemibarbus labeo*									3			3	HQ536371.1	100	99	Sp
				Pseudorasbora parva						1						1	HQ536453.1	100	98	Sp
		Perciformes	Centrarchidae	*Micropterus salmoides*	15											15	DQ536425.1	98	99	Sp
			Gobiidae	*Tridentiger brevispinis*		7	1	18	2	23	18		7	31	15	122	HQ536531.1	100	100	Sp
Arthropoda	Malacostraca	Isopoda	Asellidae	*Asellus* sp.	4								2		16	22	AY531829.1	93	89	Ge
		Decapoda	Palaemonidae	*Macrobrachium nipponense*						16						16	JN874528.1	94	99	Sp
	Maxillopoda	Sessilia	Chthamalidae	*Chthamalus challengeri*			30					3				33	EU304447.1	91	99	Sp
ETC				*Homo sapiens*				13			3					16	KF540983.1	100	99	Sp
				Number of clones	31	30	31	31	37	49	30	30	37	32	31	369				
				Number of OTUs	4	2	2	2	2	4	3	2	4	2	2	10				

Following the adoption of the identification criteria described in the Materials and Methods, we determined that the prey items were found from one phylum (Animalia) and represented three classes, six orders, and eight families based on the combined analysis of BLASTn and BOLD database searches and phylogenetic tree construction (Figure S1). Acrinopterygii comprised the largest proportion (6/10 OTUs), followed by Malacostraca (2/10 OTUs) and Maxillopoda (1/10 OTU). *Tridentiger brevispinis* was the most common (9/10 OTUs), followed by *Clupea pallasii* (8/10 OTUs) and *Asellus* sp. (3/10 OTUs). Table 1 lists the prey items identified by COI barcoding (QC: Query cover, ID: Identity, IL: Identification level, Sp: Species, Ge: Genus)

3.2. Relationship between Numbers of Clones and OTUs

In order to determine the appropriate number of clone samples needed to reach relative stability for OTU identification, the numbers of clone samples and OTUs were compared using the sequence identification information. Rarefaction analysis results are illustrated in Figure 2. The number of OTUs converged to a stable state as the number of clone samples increased. In the case that two OTUs were expected, more than 20 clones were required, which gradually increased to >25 clones and >36 clones for three and four OTUs, respectively (95% confidence). Even though three samples (C-1, C-3, and C-11) did not converge, most of the samples' OTU numbers converged as the clone number increased (Figure 2A).

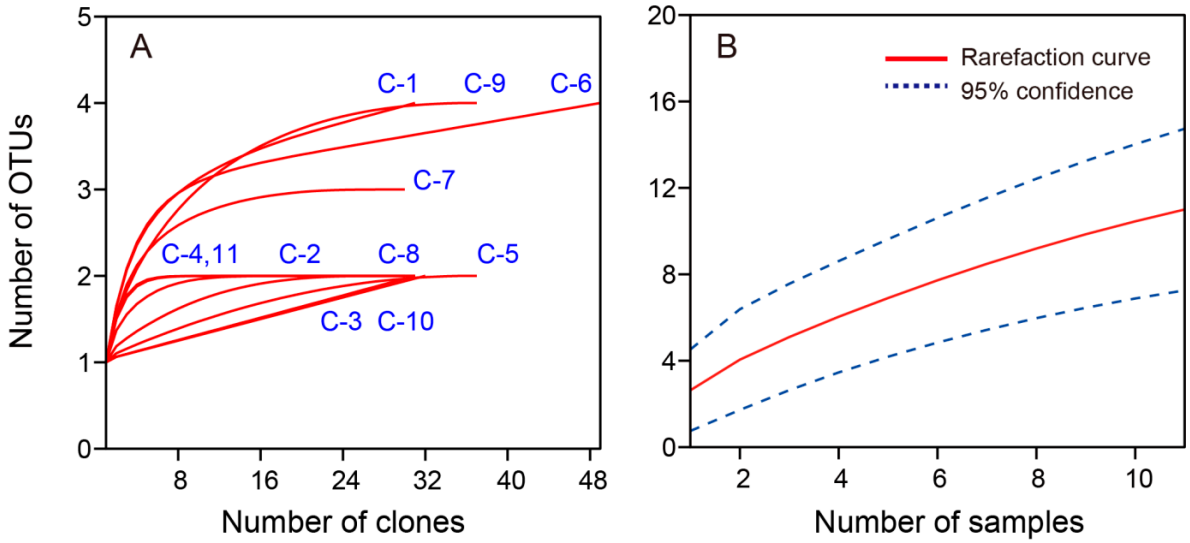

Figure 2. Rarefaction curve based on number of OTUs ((**A**) number of clones, (**B**) number of samples).

The number of OTUs in the pooled gut samples (11 total fecal samples) was compared with the number of samples in rarefaction analysis (Figure 2B). The expected number of OTUs did not converge, even though the sample number reached its maximum. This indicates that more prey items are expected, and the number of fecal samples used in the current study was not sufficient for generalizing the predation pattern of the little tern.

4. Discussion

The results of the current study emphasize that fecal materials are good sources for identifying prey species that are consumed by carnivorous avifaunal species. Using this approach, we improved prey identification resolution and produced relatively robust prey data. The prey investigation was also carried out with minimal disturbance and injury to the predators of interest.

The resolution could be based on the predation habits of the little tern. This species usually swallows its prey whole. Hard parts of the swallowed prey, such as bones from fish or the chitin carapace of arthropods, might be less digested in the little terns' guts prior

to excretion. The primer sets used in this study, LCO1490 and HCO2190, are recognized as suitable for animal species identification because of their relatively larger universality. The primers target a relatively long sequence region (i.e., 658 bps), and the hard parts of the prey might allow the primers to successfully bind to these longer regions [31]. It would be affected by the retention time of the consumed food for little tern. Therefore, we should consider it for further research.

The primer sets used in this study are not designed to detect denatured or fragmented DNA samples; therefore, the number of prey species can increase when different primer sets designed for degraded DNA are adapted. However, the advantage of COI region barcoding (i.e., the high resolution of prey identification) cannot be overlooked. Therefore, we encourage balancing the two different prey identification approaches to improve the resolution.

DNA barcoding of fecal materials has been proposed in some studies. Currently, DNA barcoding is popularly applied to fecal materials using either species-specific primer sets designed for identifying specific species (e.g., endangered or exotic species) [32] or primers that bind to relatively short sequences of DNA to prevent issues with DNA denaturation or fragmentation (e.g., 200–300 bps) [31]. Even though the former applications provide evidence of the prey species of interest, they do not reveal all of the prey species consumed. The latter approach uses universal primers to determine unknown consumed prey items [31]; however, this approach cannot guarantee species-level identification. Typically, primers targeting longer DNA sequences (generally more than 500 bps, e.g., COI) have a higher probability of facilitating species-level identification, and the successful application of COI to gut content analysis (e.g., Jo et al. [1]) supports the utility of DNA barcoding. However, this application requires relatively fresh samples, and universal primer targeting of longer DNA sequences has not completely been implemented in extremely denatured samples (such as fecal materials). This problem remains unsolved at present.

The most important issue of the study is to confirm the identified 'species.' Many studies previously utilized universal primer sets to attempt to identify the discovered OTUs as species based on sequence identity. Even though identity is high enough to confirm the OTUs as a species, this sole confirmation process is not free from sequence misreading or database insufficiency [28,29]. Therefore, it is recommended to compare the identified sequence with other sequence data obtainable from pub'lic sequence databases such as BOLD systems [1]. Regarding the importance of prey identification in food web structure determination, this dual certification scheme would be helpful, which was successfully evidenced in the current study.

The next step of prey identification is the quantification of prey stored in feces to improve the ecological understanding of predator–prey relationships. A state-of-the-art technique for this quantification is next-generation sequencing (NGS). Despite the utility of information derived from NGS, NGS is unfortunately expensive, which is an obstacle to ecological applications [1]. The second approach is to adopt quantitative PCR (qPCR) based on prey species-specific primer sets. However, this method is also problematic because it is difficult to prepare the primer sets. We suspect that counting the number of clones of each prey species may help elucidate the relative ratio of prey composition in each fecal sample. Evaluating the number of clones to understand prey consumption tendencies would be ideal for future research.

One conservation issue related to little tern distribution is human activity. Typically, the species arrives at the estuarine area in April and attempts to breed twice between May and June (or early July). They depart when monsoonal rainfall affects the breeding habitat (July or August, depending on the monsoon pattern; Jang et al. [11]). The breeding population of the species previously reached 3000–6000 individuals; however, during the study period, only approximately 200 individuals were present in the habitat, resulting in the difficulty of obtaining a sufficient number of fecal materials. We suspect that garbage removal from the estuarine area is responsible for the decrease in little tern abundance. This

activity was introduced by the borough office that was responsible for the implemented estuarine system management.

5. Conclusions

Fecal materials that originated from an avifaunal species were evaluated as a sample source for prey item investigation in this study, and prey consumed by little tern individuals were successfully identified by DNA barcoding, through the dual certification process. Even though the success of identification was largely dependent upon the predation habits of the little tern, the increased resolution of prey identification to the species level is important in predator–prey research. The current study used a small number of fecal samples to evaluate the applicability of COI barcoding to avifaunal feces, and more fecal samples are expected to convey increased information that can be used to infer the range of the prey species of little terns. Furthermore, prey items were robustly identified, as evidenced by two types of confirmation, indicating that fecal materials that inevitably suffer from severe denaturation may still be useful for prey identification.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su141911945/s1.

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