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Identification of bird species and their prey using DNA barcode on feces from Korean traditional village groves and forests (*maeulsoop*)

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A DNA barcode based on 648 bp of cytochrome *c* oxidase I (COI) gene aims to build species-specific libraries for animal groups. However, it is hard to recover full-length (648 bp) barcode gene from environmental fecal samples due to DNA degradation. In this study, we designed a new primer set (K_Bird), which amplifies a 226 bp fragment targeted an inner position of full-length COI barcode based on 102 species of Korean birds to improve amplification success, and we attempted to identify bird species from 39 avian fecal samples collected during 4 months from Jinan, South Korea. Simultaneously, we conducted a dietary analysis using a universal DNA mini-barcode (Uni_Minibar) from same fecal samples. *In silico* analysis on newly designed mini-barcode represented that genetic distances were 0.5% in species and 9.1% in genera. Intraspecific variations of 149 species out of 174 species (86%) between Korea and North America were within the threshold (5.3% threshold in this study). From environmental fecal samples collected in Jinan, we identified seven avian species, which have high similarity (99–100%) with registered COI sequences in GenBank. Eight kinds of prey species, such as moth, spider, fly, and dragonfly, were identified in dietary analysis. We suppose that our strategy applying mini-barcode for environmental fecal samples, might be a useful and convenient tool for species identification and dietary analysis for birds.

Keywords: cytochrome *c* oxidase I; DNA degradation; DNA barcode; mini-barcode; feces

Introduction

DNA barcoding using 648 bp region of the mitochondrial gene, cytochrome *c* oxidase I (COI) gene is widely used to identify bird species (Hebert et al. 2004; Yoo et al. 2006; Ward 2009; Kim et al. 2012). Up to date, above 25,000 specimens barcode sequences from nearly 3900 species (about 40% of all birds) were recorded in the Barcode of Life Data Systems (BOLD) website (http://www.barcodeoflife.org) and GenBank/EMBL/DDBJ from all over the world. These progress made barcode library as useful and valuable resources for scientists and the interested public. Genetic analysis based on DNA barcode can identify avian species from small body samples such as blood, muscle, and feathers. For example, small amount of bird tissue, blood, or feather samples collected from the damaged airplane were used to identify species involved in bird strike at airport (Yang et al. 2010; Waugh et al. 2011). In addition, there are some advantages for bird's trophic relations using feces or remaining stomach contents, which are not identifiable by morphological criteria, based on molecular approaches. In the past, many unethical analysis methods have been used to investigate the diet of birds under natural conditions by conducting stomach content analysis after killing and dissecting, the use of neck-collars, flushing the crop or stomach, and so on (Miller and McEwen 1995; Hull 1999; Exnerova et al. 2003; Moorman et al. 2007). Although retrieving remnants from feces for morphological identification is noninvasive, it is often difficult to identify prey species like insect larvae, which have well-degradable soft bodies (Oehm et al. 2011). On the other hand, dietary analysis using feces based on molecular approaches is nonlethal, noninvasive and allows identifying the dietary remains on species-specific level (Deagle et al. 2007, 2010).

In general, extracted DNA from fecal samples is difficult to recover target sequences longer than 200 bp because degraded DNA disrupt amplification of full-length barcode sequences (Hajibabaei et al. 2006; Meusnier et al. 2008). Especially, long amplification product sizes are limited that DNAs from fecal samples were well degraded by environmental factors, rain and ultra violet (UV) radiation, and enzymatic (Lindahl 1993; Oehm et al. 2011). In this reason, short-length barcodes are suggested for DNAs from fecal samples or guts (Deagle et al. 2005; Meusnier et al. 2008). Short-length (approximately 100–300 bp) barcodes showed higher success rates than full-length barcodes in amplification and enough resolutions in terms of species identification (Meusnier et al. 2008). Also, Meusnier et al. (2008) demonstrated that universal mini-barcode have 90% success (100 bp) in species resolution and 95% success (250 bp) while full-length DNA barcode provides 97% success in species resolution for all major eukaryotic groups.

There are many village groves and forests, called as *maeulsoop* (pronounced as má-ùl-soop), of the Korean

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agricultural landscape in Jinan, South Korea. In addition, almost all locations of *maeulsoops* remaining in Jinan were reported (Park and Lee 2007). Koh (2011) explained ecological roles of *maeulsoops* that "A *maeulsoop* was composed of an isolated tree or a number of trees and located neighboring a forest edge or amidst agricultural fields. Owing to these characteristics, *maeulsoops* can serve as useful corridors or stepping stones for wildlife." Furthermore, many ecologists attempted to explain importance's of *maseulsoops* facilitating local biodiversity (Park et al. 2006; Lee et al. 2007). Especially, related to bird communities in *maeulsoops*, Park et al. (2006) showed that *maeulsoops* (similar concept with Bibosoops) serve as a unique biotope providing nest sites for cavity nesters and internal movements of birds among patches in the landscape. However, there are few studies about bird species living in *maeulsoops,* previously. Furthermore, distinct dietary analysis of bird species in this region has not been studied in *maeulsoops*, yet. Park and Lee (2010) conducted comparison of species composition between two types of village groves in Korean rural landscapes and identified 34 bird species at 12 *maeulsoops* of Jinan County. However, they just classified observed birds into seven types of functional guilds according to five criteria: terrestrial insectivorous, aquatics foragers, ground foragers, granivorous, frugivorous, omnivorous, and predators.

In this study, we devised avian-specific mini-barcode (K_Bird), which is effective for bird identification, and applied universal mini-barcode (Uni-Minibar) designed by Meusnier et al. (2008) for dietary analysis from fecal samples. And, we tested the performances of two mini-barcode from unknown fecal samples collected from *maeulsoop*, simultaneously. Finally, we evaluated the possibility of application for avian dietary analysis from feces.

Materials and methods

Study site and sample collection

Based on Koh's (2011) previous study, we selected 14 study sites, except for Pyeongjangri (PJ), where all well-conserved *maeulsoops* are located around Mt. Mai in Jinan, in the middle of South Korea (E 127°25′49″, N 35°47′30″). Avian fecal samples were collected at the selected study sites bimonthly from May to September 2011. We collected primarily fecal samples on leaves and rocks to prevent contamination from soil materials. Collected fecal samples were placed in 2 ml Eppendorf tubes and frozen in liquid nitrogen directly during delivery to the laboratory. Fecal samples were stored at −80°C until extraction.

Primer design strategy for bird identification and dietary analysis

Primer sets for bird identification have been previously developed that amplify 5′ end of COI (Hebert et al. 2004). The sizes of amplified products are 749 and 751 bp. As DNA extracted from feces are susceptible to be degradation, we attempted to reduce PCR product size to approximately 200 bp and designed a new primer pair. We examined a single individual sequence from each of 102 Korean birds selected from 'DNA Barcoding Korean Birds' file in the BOLD website (www.boldsystems.org) (Ratnasingham and Hebert 2007). All sequences were aligned by using CLUSTAL W and calculated entropy values at each positions using BioEdit software to detect appropriate positions that showed lower variations (Thompson et al. 1994; Hall 1999; Figures 1, S1–2).[1] Entropy ($H(x)$) is modified from information theory defined by Claude Shannon and calculated as

$$H(x) = -\sum f(b,x)\,\ln(f(b,x))$$

where $H(x)$ is the uncertainty, also called entropy at position x, b represent a residue (out of the allowed choices for the sequence in question), and $f(b,x)$ is the frequency at which residue b is found at position x (Hall 1999). We designed a new primer pair, K_Bird_F1 & R1, considering physical and structural properties of primers from two relatively conserved sites, 163–181 and 408–431, which showing low entropy values (Table 1).

Amplified region by the newly designed primer pair had approximately 226 bp length and located on middle of full-length COI barcode gene (Figure 1). For dietary analysis from feces, we used a universal primer, Uni_Minibar_F1 & R1, designed for biodiversity analysis of eukaryotes (Meusnier et al. 2008). This primer set has an advantage for detection of prey species such as insect and fish (Meusnier et al. 2008). The amplification products were approximately 130 bp and located 5′ end of barcode region (Figure 1, Table 1).

DNA preparation, PCR amplification, and sequencing

DNA was extracted from avian feces using QIAamp® DNA Stool Mini Kit (Qiagen) according to manufacturer's protocols except for the lysis step. Fecal samples, less than 220 mg, were used for DNA extraction by manufacturer's suggestions. For sufficient homogenization, we added one or two 5 mm stainless steel beads (Qiagen) in the lysis step and mixed by vortexing for 1 min or shaking using Mixer Mill (Retsch, Germany) for 20 Hz, 1 min. PCR amplifications of COI gene for identifying bird species were carried out using newly

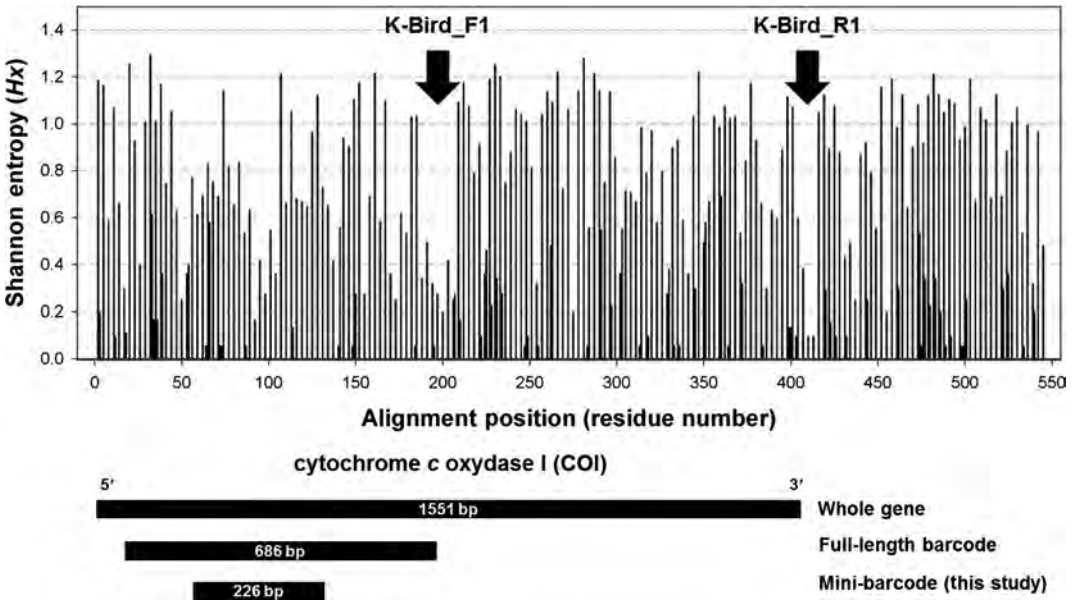

Figure 1. The Entropy plot based on aligned COI sequences of 102 Korean birds selected from 'DNA Barcoding Koran Birds' file in the Barcode of Life Data Systems (BOLD) website (www.boldsystems.org). Positions of each primer were indicated by arrow. The amplification product size and relative position with reference is indicated below the entropy plot. Total COI gene is extracted from complete mitochondrion sequences of *Anser albifrons* (accession number: NC_004539) and represented as full-length barcode is COI sequence of *A. albifrons* (accession number: EF515786) from 'DNA Barcoding Korean Birds' file.

designed primer pair, K_Bird_F1 & R1. For detection of prey species, PCR amplifications were carried out by using Uni-Minibar_F1 & R1 primer pair. In each PCR amplification, 1 μl of extracted DNA was added to 24 μl of the amplification mixture, giving rise to final concentrations of 1 × Ex *Taq* Buffer, 1.5 mM of $MgCl_2$, 10 mM of dNTP mix, 0.2 μM of each primer, 0.1 M of BSA, and 1 U of Ex *Taq* DNA polymerase (Takara, Japan), in a final volume of 25 μl. Conditions for PCR were as follows: an initial denaturation at 95°C for 2 min, 5 cycles of denaturation at 95°C for 1 min; annealing at 46°C for 1 min; elongation at 72°C for 30 s, 45 cycles of denaturation at 95°C for 1 min; annealing at 53°C for 1 min; elongation at 72°C for 30 s, and a final extension step at 72°C for 5 min. After the reactions, amplified PCR products were purified by using Expin™ Gel SV Kit (GeneAll, Korea). Purified PCR products were inserted into the pGEM®-T Easy Vector according to manufacturer's protocols (Promega, USA) and transformed into DH5α chemically competent cells. Cells were plated in Luria-Bertani agar + ampicillin medium with 40 μl of X-gal solution (2% w/v) for screening. After cloning, three to five of white-colored colonies were selected and amplified by colony PCR with M13F&M13R primer. Conditions for PCR were as follows: an initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s; elongation at 72°C for 1 min; and a final extension step at 72°C for 7 min. After the reactions, amplified PCR products were purified by using Expin™ PCR SV Kit (GeneAll, Korea). Sequencing was conducted by commercial sequencing service company (Macrogen, Korea). Each obtained sequence was identified by BLASTN analysis in the GenBank database and BOLD-identification system (IDS) online workbench provided by the BOLD website (www.boldsystems.org) (Ratnasingham and Hebert 2007). Sequence divergences were calculated using the Kimura-2-parameter (K2P) model (Kimura 1980). Genetic distance was represented with each

Table 1. Primer sequences used in this study.

Primer name		Primers sequences		Target species	Reference
K_Bird_F1	5′	CCC CAG ACA TAG CAT TYCC	3′	Birds	This study
K_Bird_R1	5′	TTG TGA TAG TGG TGG GGT TTT AT	3′		
Uni_Minibar_F1	5′	TCC ACT AAT CAC AAR GAT ATT GGT AC	3′	Universal	Meusnier et al. (2008)
Uni_Minibar_R1	5′	GAA AAT CAT AAT GAA GGC ATG AGC	3′		

taxonomic level based on sequence divergences results. Phylogenetic analysis by neighbor-joining method was conducted based on K2P model (Saitou and Nei 1987). Sequence divergences and phylogenetic analysis were conducted by using MEGA5 program (Tamura et al. 2011).

Statistical analysis

Paired *t*-test was conducted to evaluate if the distance measures are different in full-length barcode vs. mini-barcode by calculating *P* for each mini-barcode in comparison to the full-length barcode. Paired *t*-test was conducted using R software packages (stats-package), version 2.15 (R Core Team 2012) and *P* values were calculated in paired *t*-test. Genetic distances were calculated by using MEGA5 software (Tamura et al. 2011).

Results

In silico *analysis*

We first conducted an *in silico* analysis to compare COI sequence differences in species-level identification between full-length barcode and mini-barcode region amplified by newly designed K_Bird_F1 & R1 primer. The compared data-sets consisted of a combination between birds of North America including 419 specimen and birds of Korea including 255 specimen from BOLD (www.boldsystems.org). From the data sets, 510 COI sequences in 174 species which have two or more individuals were selected and calculated genetic differences based on Kimura-2-parameter (Figure 2). Genetic differences were average 0.5% within species level and 9.1% within genus-level. Most species showed low-intraspecific and high-interspecific genetic distance, however, two individual COI sequences of *Carpodacus mexicanus* were separated each other and one of this were grouped with *Tringa solitaria* in neighbor-joining (NJ) tree. Of those species comparison between intraspecific and interspecific genetic distances ($n = 83$), most species, except for *C. mexicanus* and *Sturnella magna*, had higher values of minimum congeneric distance than maximum intraspecific distance (Figure 3). The average maximum intraspecific distance within 83 species was 0.63% while the average minimum congeneric distance was 7.76% (12-fold higher). In case of *C. mexicanus*, maximum intraspecific distance was 16.8% and minimum congeneric distance was 9.8%. Intraspecific variations of 149 species out of 174 species (86%) were within the threshold that should be 10 times as much as mean intraspecific variation (5.3% threshold in this study) for the group proposed by Hebert et al. (2004). Paired *t*-test for each mini-barcode in comparison to the

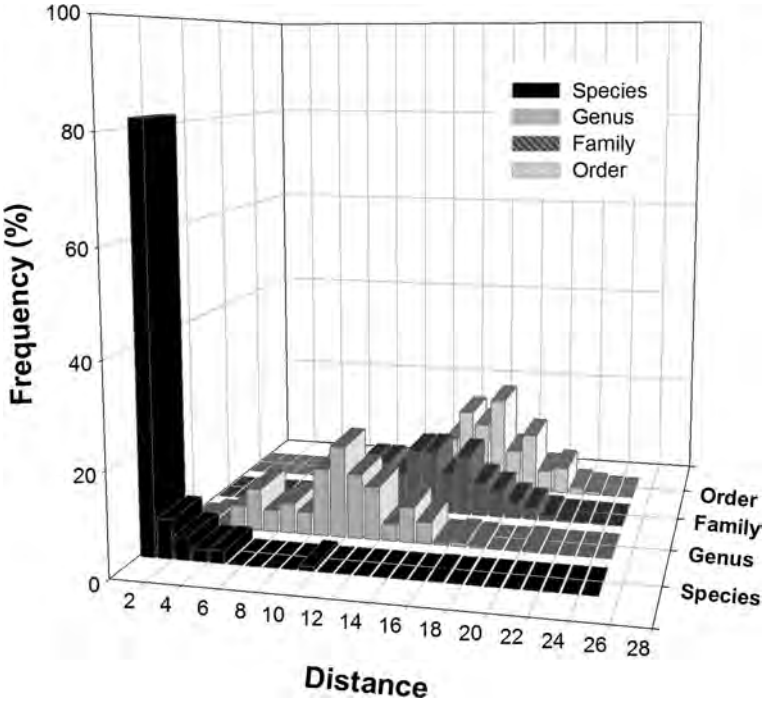

Figure 2. Distribution of genetic distances in COI among 174 bird species of Korea and North America based on Kimura-2-parameter. Pairwise comparisons between 510 COI sequences from two barcode data-set are represented with each taxonomic level.

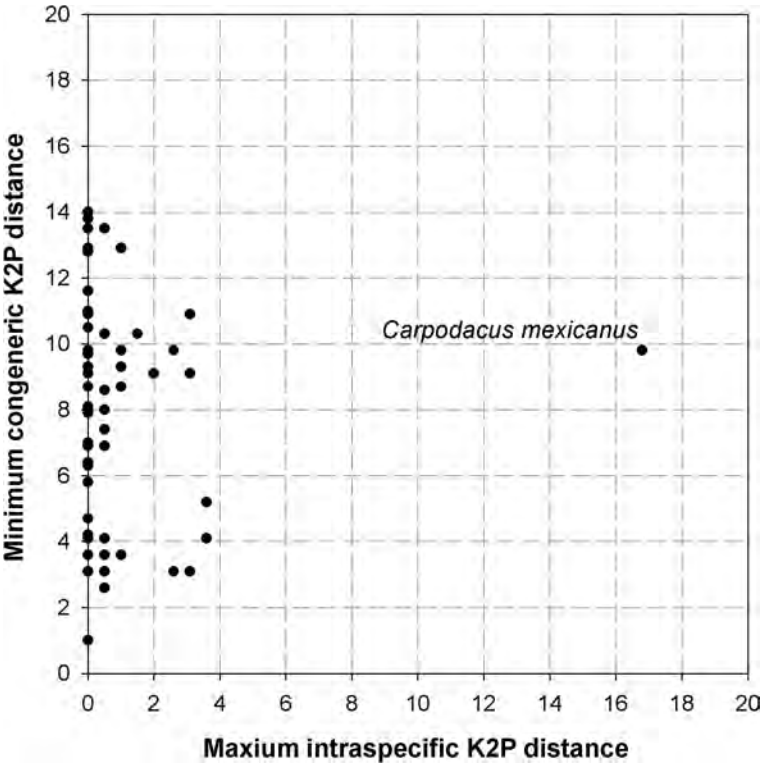

Figure 3. Comparison between intraspecific and interspecific genetic distances (K2P) for individual species based on short-length (226 bp) COI sequences corresponding mini-barcode. Each species which is possible to compare between maximum intraspecific and minimum congeneric distances were illustrated as a dot ($n = 83$).

full-length barcode showed that there were significant shifts in divergence values, which were noted in comparisons of mini- vs. full-length barcodes except to intraspecific distances in the 3′ end of mini-barcode region (Table 3). Mini-barcode amplified by K_Bird primer set showed the highest percentages of variability (47.3%) and parsimony informative sites (44.2%) and the longest amplicon sizes (226 bp) (Table 3). In comparison of genetic distance including 510 COI sequences in 174 bird species on Korea and North America, statistical analysis showed that differences in mean genetic distances were not statistically significant in comparisons of mini- vs. full-length barcodes within species level. However, significant shifts in divergence values were observed in higher taxonomic level (Table 4).

Birds identification and prey detection from environmental fecal samples

We analyzed 39 unidentified avian fecal samples from village groves and forests of the Korean agricultural landscape located in Jinan during experimental periods using two different primer set both K_Bird_F1 & R1 for bird identification and Uni_Minibar F1 & R1 for prey detection (Table 2). For bird identification, 24 out of 39 fecal samples (61.5%) were amplified. In addition, 25 out of 39 fecal samples (64.1%) were amplified for prey detection using Uni_Minibar_F1 & R1. More than one-third of fecal samples (16 specimen) were identified for both birds and prey species. Seven avian species, *Cyanopica cyanus*, *Garrulus glandarius*, *Passer montanus*, *Pica pica*, *Oriolus chinensis*, *Sturnus cineraceus*, and *Streptopelia orientalis,* were identified from amplified 24 fecal samples. All of identified seven avian species showed high similarity (99–100%) with registered COI sequences in GenBank from the BLASTN results. Two avian species of Corvidae, *P. pica* and *C. cyanus*, were always detected during the whole study period. *S. cineraceus* and *P. montanus* were detected in May and July, and *S. orientalis* were detected in July and September. *O. chinensis,* and *G. glandarius* were detected only in September.

In dietary analysis, most of COI sequences amplified from avian fecal samples belonged to one of four Order (Araneae, Diptera, Hemiptera, and Megaloptera). Three species, *Acronicta rumicis*, *Polygonia caureum*, and *Stomoxys calcitrans,* had 100% similarity from BLASTN. Seven out of 25 amplified fecal samples showed description as birds or fungi. BLASTN results showed lower similarity values (82–100%) compared to the results of bird identification.

Table 2. BLAST results of identified 25 environmental fecal samples collected from Jinan, South Korea, 2011.

No.	Sample ID	BLAST results					Primers
		Description	Query coverage (%)	*E* value	Max ident (%)	Accession	
1	May-1	*Pica pica*	100	2.00E-114	100	HQ915867	B
2	May-2	*Cyanopica cyanus*	100	4.00E-112	99	GQ481686	B
3		*Diptera* sp.	99	2.00E-23	83	JF867480	P
4		*Diptera* sp.	99	2.00E-23	83	JF867480	P
5	May-3	*C. cyanus*	100	4.00E-112	99	GQ481686	B
6		*Megaloptera* sp.	99	2.00E-28	85	HM435016	P
7	May-4	*Sturnus cineraceus*	100	8.00E-114	100	JF499168	B
8		*Diptera* sp.	98	5.00E-38	90	HM881198	P
9	May-5	*S. cineraceus*	100	2.00E-114	100	JF499168	B
10		*Diptera* sp.	99	2.00E-23	83	JF867480	P
11		*Araneae* sp.	99	3.00E-40	91	HQ983117	P
12	Jul-1	*Araneae* sp.	100	2.00E-57	99	HQ975053	P
13	Jul-2	*C. cyanus*	100	4.00E-112	99	GQ481686	B
14	Jul-3	*C. cyanus*	100	4.00E-112	99	GQ481686	B
15	Jul-4	*C. cyanus*	100	2.00E-110	99	GQ481686	B
16	Jul-5	*Streptopelia orientalis*	100	8.00E-114	100	GQ482677	B
17		*Streptopelia orientalis*	100	4.00E-59	100	GQ482677	P
18	Jul-6	*Passer montanus*	100	8.00E-114	100	GU572006	B
19		*Polietes lardarius*	98	3.00E-36	89	FJ025653	P
20		*Acronicta rumicis*	100	4.00E-59	100	HQ563400	P
21	Jul-7	*Pica pica*	100	8.00E-114	100	HQ915867	B
22		*Pica pica*	100	4.00E-59	100	HQ915867	P
23		*Diptera* sp.	99	7.00E-22	82	JF867480	P
24	Sep-1	*C. cyanus*	98	6.00E-105	98	GQ481686	B
25		*Stomoxys calcitrans*	100	4.00E-59	100	EU029768	P
26	Sep-2	*Oriolus chinensis*	100	8.00E-114	100	GQ482278	B
27		*Acronicta rumicis*	100	4.00E-59	100	HQ563400	P
28	Sep-3	*Streptopelia orientalis*	100	8.00E-114	100	GQ482678	B
29		*Streptopelia orientalis*	100	4.00E-59	100	GQ482678	P
30	Sep-4	*Pica pica*	100	1.00E-122	99	HQ915867	B
31		*Rhizopus oryzae*	100	1.00E-50	94	AY863212	P
32		*Garrulus glandarius*	100	4.00E-59	100	GQ481961	P
33	Sep-5	*Pica pica*	98	1.00E-111	100	HQ915867	B
34		*Hemiptera* sp.	99	4.00E-49	95	HM428692	P
35	Sep-6	*C. cyanus*	100	2.00E-110	99	GQ481686	B
36		*Polygonia caureum*	100	4.00E-59	100	GU372511	P
37	Sep-7	*Pica pica*	100	8.00E-114	100	HQ915867	B
38		*Polietes lardarius*	98	3.00E-36	89	FJ025653	P
39	Sep-8	*O. chinensis*	100	8.00E-114	100	GQ482278	B
40		*Hemiptera* sp.	100	1.00E-44	92	GU671578	P
41	Sep-9	*G. glandarius*	100	8.00E-114	100	GQ481961	B
42		*G. glandarius*	100	4.00E-59	100	GQ481961	P
43	Sep-10	*Streptopelia orientalis*	100	8.00E-114	100	GQ482678	B
44		*Polietes lardarius*	98	3.00E-36	89	FJ025653	P
45	Sep-11	*Pica pica*	100	2.00E-110	99	HQ915867	B
46		*G. glandarius*	100	4.00E-59	100	GQ481961	P
47		*Polietes lardarius*	98	1.00E-34	89	FJ025653	P
48	Sep-12	*Pica pica*	100	8.00E-114	100	HQ915867	B
49		*Polietes lardarius*	98	6.00E-33	88	FJ025653	P
50	Sep-13	*Pica pica*	100	6.00E-110	99	HQ915867	B
51		*G. glandarius*	100	2.00E-57	99	GQ481961	P

Note: B, K_Bird primer set; P, Uni-Minibar primer set designed by Meusnier et al. (2008).

Especially, all the sequences described as *Diptera* sp. had relatively low similarity values (82–90%).

Discussion

Through the application of newly designed mini-barcode, K_Bird, and Uni-Minibar, concurrently, we could detect seven different bird species and their preys, such as moth, spider, dragonfly, and fly, on feces collected from 14 different *maeulsoops* located in Jinan County. Especially, mini-barcode (K_Bird) had enough genetic differences between intraspecific and interspecific to discriminate bird species. These results suggest that it is possible to investigate dietary pattern of each bird species from feces effectively and allows identifying the dietary remains on species level.

To apply mini-barcode as a tool for bird identification and dietary analysis from fecal samples, it is essential to satisfy two conditions, enough resolution for species identification and high success rate of amplification. In comparisons with three different mini-barcode regions divided from full-length barcode, mini-barcodes generally provided similar percentages of variability and parsimony informative sites, although there were significant shifts in divergence values by position (Table 3). In addition, NJ analysis, most of species showed higher bootstrap values than 95% in species level, except to one species (*Anser albifrons*), which did not grouped in species level (Figure S3).[2] However, same results were obtained in NJ analysis based on full-length barcode. Especially, mini-barcode amplified by K_Bird primer set showed the highest percentages of variability (47.3%), parsimony informative sites (44.2%), and the longest amplicon sizes (226 bp) (Table 3). In comparison of genetic distance in 174 bird species on Korea and North America, differences of mean genetic distances were not statistically significant in comparisons of mini-barcode (K_Bird) vs. full-length barcodes within species level (Table 4). These results suggest that mini-barcode (K_Bird) might be a useful choice to identify bird species from fecal samples although genetic distances showed lower resolution compared to full-length barcode. In this reason, we think that the use of mini-barcode amplified by K_Bird primer set is appropriate for species identification from fecal samples of birds living in Korea.

Regarding success rates of amplification, amplicon size of our mini-barcode (226 bp) is appropriate for successful amplification from avian fecal samples. Our results showed that PCR success rates using two primer pair, K_Bird and Uni_Minibar, were about 60–65%. We assumed that 60–65% of success were not low rates considering the negative effect of environmental factors such as UV and rain. Previous studies suggest that DNA detection success in fecal samples would be improved with collection of feces which were not contaminated, fresh, and protected from sunlight and rain (King et al. 2008; Oehm et al. 2011). However, it is not always feasible to follow their suggestion in the field. In this study, to improve DNA amplification rate, we collected samples on fallen leaves or rocks under trees, which were known as resting and excreting place of birds. In addition, all of samples were collected in early morning to reduce effect of UV and weather condition. Although species resolution showed lower values compared to full-length barcode, we have demonstrated that reliable sequence information can be obtained from avian fecal samples with degraded DNA for bird identification.

Our results support that Uni-Minibar primer set is a good choice for dietary analysis from environmental avian fecal samples. Although this primer has not been designed for the purpose of dietary analysis, their amplification product sizes are about 130 bp and they target COI gene as barcode which is generally used for species-level identification in several animal groups (Meusnier et al. 2008). Moreover, one of the best

Table 3. A comparison of full-length barcode and mini-barcodes. Analysis was conducted using comparable 238 sequences from Korean birds selected from 'DNA Barcoding Korean Birds' file in the Barcode of Life Data Systems (BOLD) (www.boldsystems.org).

DNA barcode	Length (bp)	Variability (%)[a]	Parsimony (%)[b]	Percentage of intra-specific (SE)[c]	Percentage of intra-generic (SE)[d]
Full-length barcode	686	46.0	43.0	0.2 (0.0)	9.5 (0.2)
Mini-barcode (5′ end)	205	45.9	42.9	0.3 (0.0)*	10.0 (0.3)*
Mini-barcode (K_Bird)	226	47.3	44.2	0.3 (0.0)*	10.8 (0.2)*
Mini-barcode (3′ end)	212	47.2	42.0	0.2 (0.0)	8.4 (0.3)*

[a]Percentage of sites that varied in the sequences, [b]Percentage of parsimony informative sites in the alignment, [c]Average pairwise intraspecific Kimura-2-parmeter distances (Kimura 1980), [d]Average pairwise intrageneric Kimura-2-parmeter distances (Kimura 1980).
The abbreviation of 'SE' represents standard error.
*$P < 0.05$.

Table 4. Comparison of genetic divergences between mini-barcode and full-length barcode among various taxonomic levels. Data of mini-barcode (510 COI sequences of 174 species) and full-length barcode (480 COI sequences of 163 species) were selected from total 674 registered COI sequences of bird species between Korea and North America in Barcode of Life Data Systems (BOLD) website (www.boldsystems.org).

DNA barcode	Length (bp)	Comparisons within	No. of comparisons	Distance			Standard error
				Minimum	Mean	Maximum	
Mini-barcode	226	Species	718	0	0.5	16.8	0.051
		Genus	1030	1.0	9.1*	20.1	0.100
		Family	6663	4.2	13.1*	23.3	0.039
		Order	17,992	9.0	16.1*	23.6	0.018
Full-length barcode	580[a]	Species	680	0	0.8	20.4	0.096
		Genus	925	0.8	9.1	21.0	0.105
		Family	5586	2.4	13.5	21.8	0.033
		Order	15,632	11.0	16.6	21.7	0.013

[a]Comparable COI positions (580 bp), except to short-length sequences and having a lot of unknown bases, are analyzed as full-length barcode in this study.
*$P < 0.05$.

advantages is their universality. Meusnier et al. (2008) showed that Uni_Minibar primers detected a comprehensive set of taxa from all major eukaryotic groups. In addition, 90% of success was obtained with primer length of 100 bp while 97% species resolution were obtained from full-length DNA barcodes. These advantages of Uni-Minibar, short products length and enough resolution, make it useful to survey dietary organisms of avian species from fecal samples. Despite these benefits, however, some of taxonomy groups (Lepidoptera, in case of our results) could not be resolved in species level. For example, some unidentified COI sequences were matched with nine different species, *Acronicta mansueta*, *A. superans*, *A. quadrata*, *A. innotata*, *A. rumicis*, *Agriopodes fallax*, *Concana mundissima* DHJ01, *Capis curvata*, and *Pharetra rumicis*, which had same similarity values (100%) in BOLD-IDS. In addition, the identification results both BLASTN and BOLD-IDS represented just order level in case of Diptera, Hemiptera, and so on. We assumed that this result derived from a lot of unidentified barcode information registered in barcode database and genetic differences by locality. For example, more than 70% was unspecified from 1,10,371 published records founded by Diptera in BOLD database (Ratnasingham and Hebert 2007). Furthermore, more than 70% of these records were registered from just three countries, Canada, Costa Rica, and the USA. In this reason, it is essential to construct local DNA barcode database related to target dietary species such as insect, spider, and so on. Construction of local database can be helpful to identify each species exactly and increase the resolutions for dietary analysis. In addition, after determining the groups at the order level using universal primer set, the use of group-specific primer for accurate identification of dietary species can be compensated low resolution problem.

For more complete dietary analysis, it is important to detect plant species from fecal samples. Fortunately, we could not find any fragments of plant organisms such as stems, fruits, and seeds from remaining feces in lysis step under the microscope in this study. However, we could not exclude the possibility of feeding plant organisms because all bird species detected in this study are known as omnivorous. Our strategy, used Uni_Minibar primer set for dietary analysis, has a limitation that it could not detect plant DNAs from fecal samples. Recently, the Consortium for the Barcode of Life-Plant Working Group has recommended two loci (rbcL + matK) as a potential barcode candidate for plant species (Vijayan and Tsou 2010). Constructing plant barcode based on these two loci and designing plant-targeted primer set with enough resolution and amplification success rates, would improve detection of plant species in avian fecal samples.

Of course, our strategy contained some limitations, which is to be supplemented for accurate bird identification and prey detection. Our mini-barcode primer pair, K_Bird_F1 & R1, was designed based on each of 102 Korean birds registered in the BOLD website (www.boldsystems.org). In this study, we added IUPAC code for polymorphic nucleic acids inside forward primer to amplify COI sequences of the Korean birds for greater efficiency. However, we should consider the possibility that some of bird species can not be amplified by our mini-barcode primer set. In the study of Hebert et al. (2004), three different reverse primers were used for amplification. In cases where first primer

pair failed, an alternate reverse primer was generally combined with forward primer. Although this strategy requires a lot of effort during PCR amplification, it can be used as a way to compensate amplification problems.

It is important to note the possibility that results of identification can be differed between selected mini-barcode (K_Bird) region and other region excluded in this study. In the study of Hajibabaei et al. (2006), they compared species-level resolution between full-length barcodes and two mini-barcodes have different length and position about microgastrine parasitic wasps. They found that small size mini-barcode (135 bp) showed different discrimination ability by position. However, the NJ analysis in this study indicates that all of mini-barcode regions including both sides of excluded regions produce species-level resolutions as effective as that of full-length barcodes on comparable 238 COI sequences of Korean birds, fortunately (Figure S3).[3] We assume that this result can be interpreted as due to similarly distributed variation sites affecting the resolution (Table 3). Percentage of site that varied in the sequences and parsimony informative sites in the alignment showed similar proportion in all barcode regions. From these results, we assumed that the possibility of different identification results according to barcode regions would be very low in case of Korean birds.

Our strategy applying mini-barcode for environmental fecal samples provides a useful and convenient tool for birds species identification and dietary analysis concurrently although there are some limitations as mentioned above. Application of mini-barcode for bird identification and dietary analysis can be helpful to understand different use of prey for each bird species according to geographical differences in Korean agricultural landscape. In addition, compared to previous methods, it is possible to investigate the change of prey preferences according to the seasonal change, especially in breeding season. The current DNA barcode database is still being constructed and accumulation of barcode information would improve resolution of identification. Primer sets used in this study may be a useful addition for other approaches such as pyrosequencing and microarray. We expect that our strategy would be progress DNA barcoding in fields of trophic relations and food-web studies focused on birds.

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Notes

1. Supplementary material can be found by clicking on the Supplementary Content tab at http://dx.doi.org/10.1080/19768354.2012.720939.
2. See note 1 above.
3. See note 1 above.

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