Mol. Cells *30*, 295-301, October 31, 2010
DOI/10.1007/s10059-010-0118-8

Molecules and Cells



# A DNA Microarray for Identification of Selected Korean Birds Based on Mitochondrial Cytochrome c Oxidase I Gene Sequences

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**DNA barcoding with the gene encoding cytochrome *c* oxidase I (COI) in the mitochondrial genome has been proposed as a standard marker to identify and discover animal species. Some migratory wild birds are suspected of transmitting avian influenza and pose a threat to aircraft safety because of bird strikes. We have previously reported the *COI* gene sequences of 92 Korean bird species. In the present study, we developed a DNA microarray to identify 17 selected bird species on the basis of nucleotide diversity. We designed and synthesized 19 specific oligonucleotide probes; these probes were arrayed on a silylated glass slide. The length of the probes was 19-24 bps. The *COI* sequences amplified from the tissues of the selected birds were labeled with a fluorescent probe for microarray hybridization, and unique hybridization patterns were detected for each selected species. These patterns may be considered diagnostic patterns for species identification. This microarray system will provide a sensitive and a high-throughput method for identification of Korean birds.**

## INTRODUCTION

DNA barcoding with 5′-end DNA sequences of the cytochrome *c* oxidase I (*COI*) gene in the animal mitochondrial genome is a useful and effective technique to identify and discover animal species (Nijman and Aliabadian, 2010; Rasmussen et al., 2009). The findings of 2 previous studies concerning North American birds showed that individuals could be assigned to a species in 94% of the cases, and that a threshold of 10 times the mean intraspecific variation can be used to identify potential new species (Hebert et al., 2004; Kerr et al., 2007). The results of our previous study (Yoo et al., 2006) supported that DNA barcoding with *COI* sequences obtained from 92 species of Korean birds enabled effective discrimination of species boundaries with a slight overlap of intra- and interspecific genetic distances. The DNA barcode, the *COI* gene, for identifying animal species is well established. However, the search for a DNA barcode for indentifying plant species has proven more challenging (Gonzalez et al., 2009). The *COI* gene among terrestrial plant species is highly invariant, and therefore, this gene is unsuitable as a DNA barcode. The Plant Working Group of the Consortium for the Barcode of Life (CBOL) recommended *rbcL* and *matK* genes as DNA barcodes for plant species (Hollingsworth et al., 2009). The *COI* gene of blowflies is also the most common molecular marker for calculation of postmortem interval (PMI) in forensic studies (Nelson et al., 2007).

Microarray technologies, which involve hybridization of short specific probes to target DNA and subsequent detection of the hybridization signal, have been widely used as diagnostic tools for genetic diseases in gene-expression studies. In particular, many microarray datasets for detection of specific viruses and pathogens have been developed (Engel et al., 2010; Lee et al., 2010b; Zhang et al., 2010). To date, very little attention has been focused on the use of microarray technologies to identify species in biodiversity-monitoring studies. In a previous study (Pfunder et al., 2004), microarray diagnostic tools for identifying a few species of voles and shrews were designed; these tools were used to develop a mammalian chip. Hajibabaei et al. (2007) designed probes from genes encoding COI and mitochondrial cytochrome *b* to discriminate species by using a variety of mammalian specimens. In food industry, DNA barcodes can be useful for prevention of commercial fraud and investigation of food-related illness (Lowenstein et al., 2009; Yancy et al., 2008). Recently, a commercial microarray was developed by Greiner Bio One for detection of 8 different animal species in the food product. This microarray is based on species-specific differences between the sequences of the gene encoding cytochrome *b*.

In the present study, we developed a microarray to identify 17 selected Korean species on the basis of barcode records of these birds that we previously determined. We selected these birds because of their importance in biodiversity monitoring and in epidemiological studies of avian influenza. It is impossible to identify species by using DNA sequencing technology with

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Received January 11, 2010; revised July 15, 2010; accepted July 19, 2010; published online September 2, 2010

**Keywords:** birds, cytochrome *c* oxidase I, DNA barcoding, Korea, microarray, molecular probes

mixed fecal samples because of the double peaks in the sequencing data. However, spotted microarray with species-specific probe is a useful tool for species identification.

## MATERIALS AND METHODS

### Selection of candidate probes

To select target species-specific probes, *COI* sequences used in our previous study (Yoo et al., 2006) were aligned using the MegAlign software (DNASTAR, USA). Among the species examined, we selected 17 target species according to the commonality of their overall presence in the environment and risk of a bird strike. We also selected the species for which records of at least 2 specimens were obtained in our previous study. For the discovery and design of species-specific oligonucleotide probes, we searched diagnostic regions throughout the gene. In these regions, candidate probes showing diagnostic differences between target species and non target species have been chosen. The 19 selected oligonucleotide probes were less than 24-bp long, and their melting temperatures (Tm) were between 49°C and 62°C (Table 2).

### Oligonucleotide microarray fabrication

Species-specific probes with a C6 5′-amino group and a poly T (15 bps) (Metabion, Germany) were synthesized. To confirm hybridization of the fluorescent-labeled polymerase chain reaction (PCR) product, we designed a position marker having no sequences that matched with those within the bird *COI* sequences. The concentration of each probe solution was adjusted to 100 pmol/μl; thereafter, 10 μl of this solution was diluted with 10 μl of spotting buffer containing 3× standard saline citrate (SSC) and 0.3 M betaine. The prepared probes contained in a 384-well plate were arrayed onto a silylated slide glass (Cell Associate, USA) at 25°C and 60% humidity. The spotted microarray was washed with 0.1% sodium dodecyl sulfate (SDS) for 5 min and then incubated in 250 ml of sodium borohydride solution ($NaBH_4$, 0.625 g; phosphate-buffered saline [PBS], 187.5 ml; and ethanol, 62.5 ml) for 5 min. Subsequently, the microarrays were washed twice by using sterile double-distilled water ($ddH_2O$) for 5 min and then centrifuged at 800 rpm for 5 min. All these steps were performed at room temperature.

**Table 1.** Cy3 labeling and sequences of PCR primers using in this study

| Primer | Cy3 labeling[a] | Sequence (5′ → 3′) |
|---|---|---|
| BirdF1 | X | TTCTCCAACCACAAAGACATTGGCAC |
| BirdR1 | O | ACGTGGGAGATAATTCCAAATCCTG |
| BirdR2 | O | ACTACATGTGAGATGATTCCGAATCCAG |
| BirdF4 | X | AACCAACCACAAAGACATTGG |
| BirdR4 | O | CCATGTAGCCGAATGGTTCT |

[a]O and X represent Cy3 labeled primer and non Cy3 labeled primer, respectively

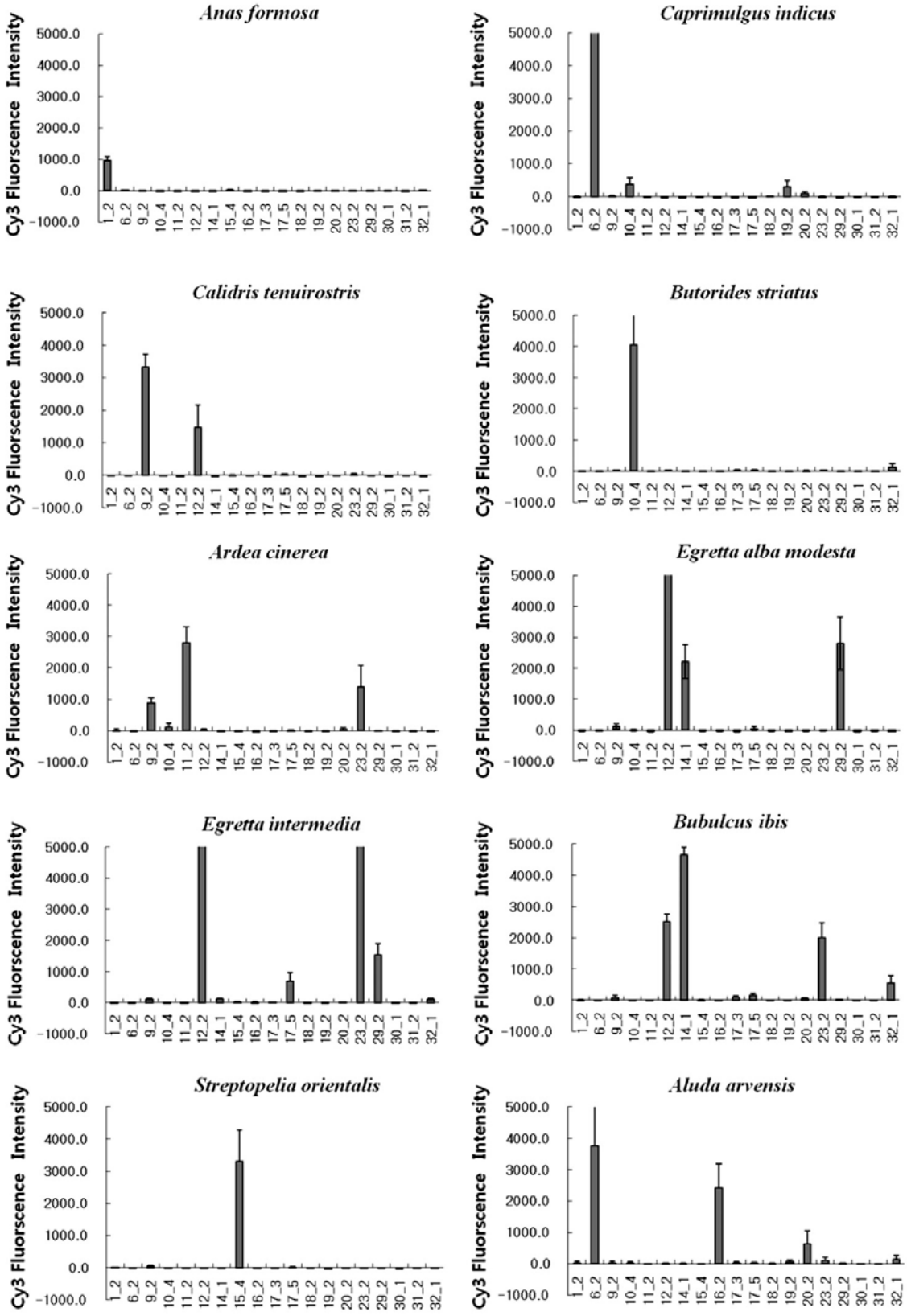
### Genomic DNA preparation, PCR amplification, and fluorescent labeling

Each species-specific probe was tested against genomic DNA of the target and non-target species. Genomic DNA was extracted using an AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea). The concentration of the genomic DNA was between 10 and 100 ng/μl. For identification of the target species, the target sequence of the *COI* gene was amplified using PCR, and then, Cy3-labeled PCR product was hybridized on the microarray. The PCR product was amplified in 20 μl of reaction mixture containing 5 μl of sterile $ddH_2O$, 10 μl of 2× DyeMix

**Table 2.** Location, Tm, CG ratio and sequence of final probes

| Probes | Sequence | Probe location | Tm (°C) | CG ratio (%) |
|---|---|---|---|---|
| 1_2 | 5′-NH2-T(15)TTCCTTCTACTACTCGCCTCATC-3′ | 265-287 | 53.41 | 47.82 |
| 6_2 | 5′-NH2- T(15)CTCCTCTCCCTCCCCGTTTTAGC-3′ | 526-548 | 62.07 | 60.86 |
| 9_2 | 5′-NH2- T(15)CGTCCTACTCTTACTCTCCCTTC-3′ | 516-538 | 52.15 | 52.17 |
| 10_4 | 5′-NH2- T(15)CCGTCTTAATCACTGCCGT-3′ | 500-518 | 52.27 | 52.63 |
| 11_2 | 5′-NH2- T(15)CATCATTCATACTTCTTCTAGCC-3′ | 260-282 | 54.56 | 30.43 |
| 12_2 | 5′-NH2- T(15)TACCGCTGTCTTACTCTTACTCT-3′ | 510-532 | 48.86 | 43.47 |
| 14_1 | 5′-NH2- T(15)CATCATTTATACTCCTACTAGCC-3′ | 270-292 | 60.23 | 56.52 |
| 15_4 | 5′-NH2- T(15)TTTTCTCTCCACCTCGCTGGTAT-3′ | 382-404 | 57.85 | 47.82 |
| 16_2 | 5′-NH2- T(15)ATCTTCTCGTTACATCTGGCAGGC-3′ | 389-412 | 59.51 | 50.00 |
| 17_3 | 5′-NH2- T(15)ATTTTCTCACTACATCTAGCAGGT-3′ | 379-402 | 56.90 | 52.17 |
| 17_5 | 5′-NH2- T(15)TCCTGTCCTTGCTGCTGGAATTA-3′ | 537-559 | 59.57 | 47.82 |
| 18_2 | 5′-NH2- T(15)AGTACTGCTTCTTCTCTCCCTAC-3′ | 516-538 | 49.65 | 47.82 |
| 19_2 | 5′-NH2- T(15)ACTACTGCTCCTGTCGCTACCCG-3′ | 519-541 | 60.48 | 60.86 |
| 20_2 | 5′-NH2- T(15)ACTAACCGATCGCAATCTAAACA-3′ | 567-589 | 54.53 | 39.13 |
| 23_2 | 5′-NH2- T(15)TCCTCTCACTCCCAGTTCTCGCT-3′ | 527-549 | 60.19 | 56.52 |
| 29_2 | 5′-NH2- T(15)TCTTCTCACTTCACCTGGCTGGT-3′ | 398-420 | 58.74 | 52.17 |
| 30_1 | 5′-NH2- T(15)ACTTTCTTTACCCGTTCTAGCCG-3′ | 528-550 | 56.83 | 47.82 |
| 31_2 | 5′-NH2- T(15)CCACCTGCCCTTTCTCAAT-3′ | 357-475 | 53.63 | 52.63 |
| 32_1 | 5′-NH2- T(15)TGTCATTACCCGTACTAGCCGCT-3′ | 489-511 | 58.64 | 52.17 |
| PM[a] | 5′-NH2- T(15)CATCCCCCTGGGACTGGAGT-3′ | - | 59.42 | 65.0 |

[a]PM: position marker, non-homologous sequence for 17 avian species


Anas formosa
Caprimulgus indicus
Calidris tenuirostris
Butorides striatus
Ardea cinerea
Egretta alba modesta
Egretta intermedia
Bubulcus ibis
Streptopelia orientalis
Aluda arvensis
Cy3 Fluorscence Intensity
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
-1000.0
1_2 6_2 9_2 10_4 11_2 12_2 14_1 15_4 16_2 17_3 17_5 18_2 19_2 20_2 23_2 29_2 30_1 31_2 32_1

(Continued)

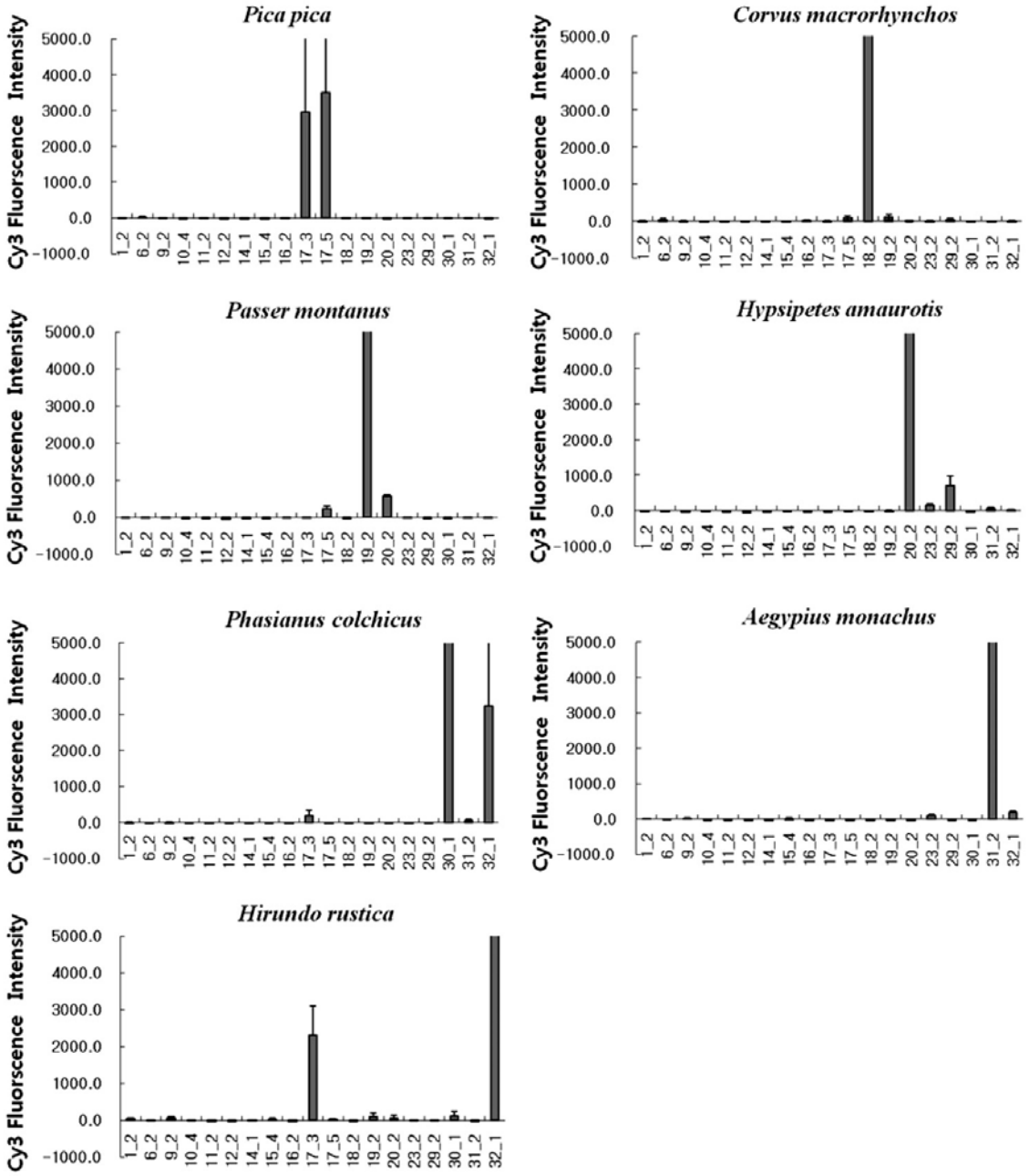



**Fig. 1.** Signal intensity plots of 19 candidate probes for 17 avian species. Signal pattern of each target species is distinguishable.

(Enzynomics, France), 0.5 μl of each primer (10 pmol), and 4 μl of template DNA by using cycling conditions as follows: denaturation for 5 min at 95°C; 40 cycles of 30 s at 95°C, at 52°C, and at 72°C; final extension for 7 min at 72°C. The oligonucleotide sequences of the 5 primers used to amplify the genomic DNA of the target bird species are presented in Table 1. For fluorescent labeling, *BirdR1, BirdR2,* and *BirdR4* were synthesized using Cy3 at the 5′ end (Metabion, Germany). The end-labeled target method is widely used to identify viral and bacterial species, because it is simple to use, and analysis of the results is easy (Diaz and Fell, 2004; Wilson et al., 2002).

**Hybridization and scanning**

The PCR products of the target and non-target species were denatured for 3 min at 99°C by using a thermal cycler (MJ Research, USA). Thereafter, 10 μl of the denatured PCR product was mixed with 90 μl of hybridization buffer (0.3% sarcosyl, 3× SSC). The mixture was hybridized to the oligonucleotide arrays; this procedure was performed for 1 h at 55°C in a hybridization oven (FINEPCR, Korea). After hybridization, the oligonucleotide arrays were washed for 5 min at room temperature with 250 ml of 0.1% SDS and 1× SSC. Finally, these arrays were washed with 0.1× SSC at room temperature for 1 min and then centrifuged at 800 rpm for 5 min.

**Table 3.** Probe signal pattern of target and non target avian species

| | **Target species** | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| probes | *Anas formosa* | *Caprimulgus indicus* | *Calidris tenuirostris* | *Butorides striatus* | *Ardea cinerea* | *Egretta alba modesta* | *Egretta intermedia* | *Bubulcus ibis* | *Streptopelia orientalis* | *Alauda arvensis* | *Pica pica* | *Corvus macrorhynchos* | *Passer montanus* | *Hypsipetes amaurotis* | *Phasianus colchicus* | *Aegypius monachus* | *Hirundo rustica* |
| 1_2 | ○ | | | | | | | | | | | | | | | | |
| 6_2 | | ● | | | | | | | | ◐ | | | | | | | |
| 9_2 | | | ◐ | | ○ | | | | | | | | | | | | |
| 10_4 | | | | ● | | | | | | | | | | | | | |
| 11_2 | | | | | ◐ | | | | | | | | | | | | |
| 12_2 | | | ◐ | | | ● | ● | ◐ | | | | | | | | | |
| 14_1 | | | | | | ◐ | | ◐ | | | | | | | | | |
| 15_4 | | | | | | | | | ◐ | | | | | | | | |
| 16_2 | | | | | | | | | | ◐ | | | | | | | |
| 17_3 | | | | | | | | | | | ◐ | | | | | | ◐ |
| 17_5 | | | | | | | ○ | | | | ◐ | | | | | | |
| 18_2 | | | | | | | | | | | | ● | | | | | |
| 19_2 | | | | | | | | | | | | | ● | | | | |
| 20_2 | | | | | | | | | | ○ | | | ○ | ● | | | |
| 23_2 | | | | | ◐ | | ● | ◐ | | | | | | | | | |
| 29_2 | | | | | | ◐ | ◐ | | | | | | | ○ | | | |
| 30_1 | | | | | | | | | | | | | | | ● | | |
| 31_2 | | | | | | | | | | | | | | | | ◐ | |
| 32_1 | | | | | | | | ○ | | | | | | | ● | | ● |

| | **Non target species** | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Non target species | *Anas acuta* | *Cyanopica cyana* | *Garrulus glandarius* | *Asio otus* | *Limosa lapponica* | *Oriolus chinensis* | *Anas clypeata* | *Phoenicurus auroreus* | *Scolopax rusticola* | *Synthliboramphus antiquus* | *Falco subbuteo* | *Ninox scutulata* | *Anser albifrons* | *Gallinula chlororpus* | *Anas crecca* | *Bubo bubo* | *Emberiza rustica* | *Larus crassirostris* | *Paradoxomis webbiana* | *Picus canus* | *Otus scops* | *Dendrocopos kizuki* |
| 1_2 | | | | | | | | | | | | | | | | | | | | | |
| 6_2 | | | | | | | | | | | | | | | | | | | | | |
| 9_2 | | | | | | | | | | | | | | | | | | | | | |
| 10_4 | | | | | | | | | | | | | | | | | | | | | |
| 11_2 | | | | | | | | | | | | | | | | | | | | | |
| 12_2 | | ● | ◐ | | | | | | | | | | | | | | | | | | |
| 14_1 | | | | | | | | | | | | | | | | | | | | | |
| 15_4 | | | | | | | | | | | | | | | | | | | | | |
| 16_2 | | | | | | | | | | | | | | | | | | | | | |
| 17_3 | | | | | | | | | | | | | | | | | | | | | |
| 17_5 | | | | | | | | | | | | | | | | | | | | | |
| 18_2 | | | | | | | | | | | | | | | | | | | | | |
| 19_2 | | | | | | | | | | | | | | | | | | | | | |
| 20_2 | | | | | | | | | | | | | | | | | | | | | |
| 23_2 | | ◐ | | ◐ | ◐ | | | | | | | | | | | | | | | | |
| 29_2 | | | | | | | | | | | | | | | | | | | | | |
| 30_1 | | | | | | | | | | | | | | | | | | | | | |
| 31_2 | | | | | | | | | | | | | | | | | | | | | |
| 32_1 | | | | | | | | | | | | | | | | | | | | | |

* ● target signal intensity > 5000, ◐ target signal intensity > 1000, ○ target signal intensity > 500

### Image analysis

The hybridization signal was detected using the microarray scanner GenePix 4000B (Axon Instrument, USA) at a photomultiplier tube (PMT) gain of 600 and 100% laser power. Thereafter, the fluorescence intensity was analyzed using the GenePix Pro 4.1 software (Axon Instrument, USA). The local background intensity was subtracted from the median value of the Cy3 signal for each spot.

## RESULTS AND DISCUSSION

### Species and probe selection

To identify the target species and candidate probes, we aligned 149 *COI* sequences from 39 avian species. We excluded the avian species that possess only 1 *COI* sequence, because we could not obtain the information of intraspecific sequence variation. The numbers of *COI* sequences and DNA samples of the target and non-target species are listed in supplementary data 1. We analyzed 69 potential probes and selected 19 candidate probes that exhibited high species specificity and signal intensity (Table 2). We also selected probes that exhibited more than 2 nucleotide substitutions in the same diagnostic region (Lee et al., 2004). Thus, 19 candidate probes were selected. The length of the probes was less than 19-24 bps, with Tm between 49°C and 62°C and GC content of 30-61% (Table 2). To prepare probes with similar Tm, candidate probes with lengths 23-24 bps were selected. However, the length of the probes 10_4 and 31_2 (each 19-bp long) had to be decreased so that Tm of these probes was similar to that of the other probes. The Tm values for all the probes were between 49°C and 62°C. Among the 19 probes, the GC content of 16 candidate probes was between 48% and 61%, but that of the remaining 3 probes (11_2, 12_2, and 20_2) was between 30% and 43%.

### Signal intensity of the candidate probes

When the PCR products of the target species were hybridized to the target probes, a signal intensity of more than 500 (arbitrary units) was obtained. The position of a probe within a target sequence is an important factor for obtaining high signal intensity (Peytavi et al., 2005). In the present study, because reverse primers were labeled with Cy3, we selected all probes as close as possible to the 3′ end of the *COI* region. We selected 19 specific probes that exhibited more than 2-base mismatch with the *COI* sequences of the species, because probes with a single-base mismatch exhibited a low discriminating power. The signal intensity was mainly influenced by the position of the fluorescent label in the PCR product, length of the probe, and base composition of the nucleotides. In particular, the position of a label rather than its binding efficiency is a crucial factor that affects the signal intensity (Zhang et al., 2005). The cross-hybridization is caused by a sequence match within the probe region. To increase the discriminating specificity, we selected candidate probes that had mismatched bases in the central region (Tambong et al., 2006).

The fabricated microarray was tested using PCR products of the target and non-target avian species. In addition, the candidate probes on the microarray were analyzed in triplicates, and the signal intensity was found to exhibit species-specific patterns (Fig. 1).

### Target species discrimination

In the present study, 1 or 2 specific probes were sufficient to identify the target species among the species examined. Recently, closely related sister species of birds could be identified from the species-specific sequence of the *COI* gene (Tavares and Baker, 2008). The probe signal patterns of 17 target species were clearly discriminated from those of the 4 non-target species (*Cyanopica cyana, Garrulus glandarius*, *Asio otus,* and *Limosa lapponica*). None of the candidate probes yielded signals in the case of 18 non-target species (Table 3). Although 10 candidate probes (6_2, 9_2, 12_2, 14_1, 17_3, 17_5, 20_2, 23_2, 29_2, and 32_1) were specific for multiple target species, these species could be distinguished using extra probes (Table 3). For example, 6_2 probe was selected to discriminate *Caprimulgus indicus*; however, this probe also yielded signals in the case of *Alauda arvensis*. However, 16_2 probe was specific only to *Al auda arvensis* among 39 avian species. Therefore, the 2 species could be discriminated using a combination of the 2 probes.

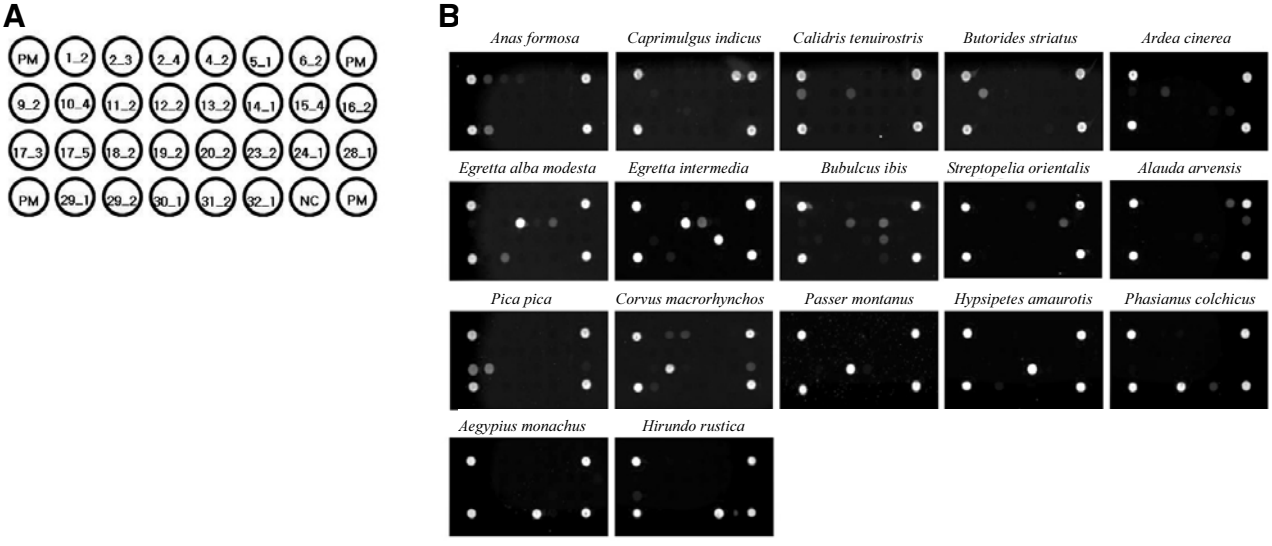



**Fig. 2.** Hybridization patterns of spotted probes on the microarray. (A) Layout of microarray for avian species identification. PM and NC indicate position marker and negative control, respectively. (B) Hybridization images for species identification of 17 avian species.

Furthermore, 4 out of 19 probes (11_2, 16_2, 18_2, and 30_1) were considered diagnostic for 1 among the 39 avian species. The patterns of hybridization for the 17 target species were also clearly discriminated from each other (Fig. 2). By using DNA barcoding with fecal samples of wild birds, isolation of avian influenza virus and identification of the host can be simultaneously accomplished (Lee et al., 2010a). This DNA chip based on DNA barcoding in birds will provide an efficient surveillance system for avian influenza virus and aid monitoring of avian influenza virus transmission through migratory birds if more comprehensive bird species should be included.

In conclusion, we could accurately identify 17 Korean birds by using a microarray with a specific hybridization pattern. Except for a few species that share the same *COI* sequences or are newly discovered, an oligonucleotide microarray based on *COI* sequences is an accurate and a rapid method for high-throughput identification of animal species. A search for candidate probes based on taxonomically broader sequence alignment and an attempt to test DNA from non-target species for the validity of the DNA chip will provide an insight into the construction of a more comprehensive avian chip. As more comprehensive records of *COI* barcodes become available, this system should find detection of species-specific oligonucleotides that have the optimal base composition and serve as an even more accurate and sensitive identification method.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

## ACKNOWLEDGMENT

This work has been supported by a grant from the Eco-Technopia 21 Project funded by the Ministry of Environment of Korea (2008-05002-0065-0).

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