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Discrimination of Animal Species Using Polymorphisms of the Nuclear Gene *Zinc Finger Protein 238*

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We screened 3750 single exonic genes listed in the intronless genes in the eukaryotes (SEGE) database and performed bioinformatic analyses to identify candidate genes for new species-specific markers. A set of PCR primers for the conserved regions of *ZNF238* was developed and used to amplify the 823 bp DNA fragment. We compared nucleotide variations of the PCR products among 20 species plus two subspecies of animals, which led to the identification of interspecies nucleotide variations. To establish a simple method for the analysis of species-specific DNA polymorphisms using *ZNF238*, we developed a PCR-RFLP method using *Hha*I and *HpyCH4*IV restriction enzymes for 13 species. For the remaining species, the direct sequencing of PCR products provided additional SNPs, enabling precise species classification. As a result, we report here that a new nuclear DNA marker, *ZNF238*, can be used to increase the accuracy of species identification among euteleostomi (bony vertebrates).

KEYWORDS: Species discrimination; PCR-RFLP; *ZNF238*; nuclear DNA

INTRODUCTION

Accurate species identity determination may be important in forensic science, food safety, and other fields. The idea of using species-specific DNA sequences for species determination was introduced as early as the 1970s (*1*, *2*). Due to the availability of genome-level DNA sequence information from diverse species, many studies have attempted to establish simple and accurate methods for classifying species information from unknown origins (*3*).

The mitochondrial genome has advantages in species identification and phylogenetic analysis, including a high copy number within the cell (*4*) and higher polymorphism generation than the nuclear genome, due to its faster evolution (*5*). Therefore, the most frequently studied genes in animal species identification have belonged to the mitochondrial genome regions, including *Cytochrome b* (*6*), *subunits of cytochrome c oxidase* (*7*), *D-loop* (*8*), and *12S* (*9*) and *16S rRNA* (*10*). In nuclear genes, *internal transcribed spacers 1-rDNA* (*11*), *recombination activation gene* (*12*), *introns of actin gene* (*13*), *myoglobin* (*14*), and *β-fibrinogen genes* (*15*) have been tested for species identification. However, nuclear genes are used less frequently in actual application, because the use of any single nuclear gene alone has proven to be insufficient for the classification of vertebrate animal species. This is due to the difficulty in finding consistent interspecies DNA polymorphisms.

In this study, we sought to identify a nuclear gene that can be used to determine the species identity more efficiently as a single locus system from nuclear DNA, even for closely related vertebrate species. We identified a region from *ZNF238* that is highly conserved among different animal species and yet contains species-specific SNPs. The nuclear gene *ZNF238* is a common gene among euteleostomi (http://www.ncbi.nlm.nih.gov/homologene) and encodes zinc finger protein 238, which acts as a transcriptional activator or repressor and is involved in chromatin assembly (*16*). We show that *ZNF238* can be used as a novel nuclear DNA marker system for the species identification of bony vertebrates.

MATERIALS AND METHODS

DNA Extraction. To test the specificity of the technique, the following numbers of unrelated specimens were tested: 10 dogs (*Canis lupus familiaris*), 6 wolves (*Canis lupus chanco*), 4 raccoons (*Nyctereutes procyonoides*), 10 pigs (*Sus scrofa domesticus*, 4 breeds), 3 wild boars (*Sus scrofa coreanus*), 8 mice (*Mus musculus*, 4 strains), 10 horses (*Equus caballus*), 5 rabbits (*Lepus brachyurus*), 10 cattle (*Bos taurus*, 2 breeds), 4 Chinese water deer (*Hydropotes inermis*), 2 goats (*Capra aegagrus hircus*), 4 western roe deer (*Capreolus capreolus*), 8 Formosan deer (*Cervus nippon taiouanus*), 10 sheep (*Ovis aries*), 10 kangaroos (*Macropus rufus*), 10 ducks (*Anas platyrhynchos*), 10 chickens (*Gallus gallus*), 5 pheasants (*Phasianus colchicus*), 3 vultures (*Aegypius monachus*), 4 falcons (*Falco peregrinus japonensis*), 3 elephants (*Loxodonta africana*), and 3 hippopotamuses (*Hippopotamus amphibius*). Animal tissues were obtained either from the

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conservation genome resource bank for Korean wildlife (http://www.cgrb.org), Seoul Zoo (http://grandpark.seoul.go.kr), local farms, local meat stores, and other researchers. Genomic DNA was prepared from tissue samples by a simple lysis method (*17*).

PCR Primers and Amplification. The nucleotide sequence information of *ZNF238* was obtained from Genbank for six species (*Homo sapiens*, NM_205768.2; *Bos taurus*, NM_001078016.1; *Equus caballus*, XM_001492205.2; *Mus musculus*, NM_001012330.1; *Canis lupus familiaris*, XM_547497.2; *Gallus gallus*, XM_426137.2). Nucleotide sequence alignments were performed with the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2), and PCR primers were designed from a highly conserved region of *ZNF238* using the primer designer V 2.0 (Scientific & Educational Software, USA). The primer sequences were 5′-ACC TGT CTG TCA AGT CCA GCC T-3′ and 5′-TCT TCT AAG GTC CAG TCT CTG A-3′ for the forward and reverse primers, respectively. PCR consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 66 °C for 45 s, and product extension at 74 °C for 45 s in a Thermocycler 3000 (Biometra, Germany). A final extension step was performed at 94 °C for 7 min. PCR amplifications were performed in a total volume of 15 μL, containing 50 ng of genomic DNA, 0.3 μM of each primer, 200 μM of dNTPs, and 0.5 unit of Supertherm DNA polymerase (LPI, U.K.) in a PCR reaction buffer (1.5 mM $MgCl_2$).

PCR-RFLP. Restriction enzymes for RFLP analysis were selected on the basis of the analysis results of restriction enzyme sites using webcutter V2.0 (http://rna.lundberg.gu.se/cutter2) or NEBcutter V2.0 (http://tools.neb.com/NEBcutter2). The PCR amplicon of *ZNF238* was digested with 10 units of *Hha*I (New England Biolabs, USA) or *HpyCH4*IV (New England Biolabs, USA) in a final volume of 20 μL for 3 h at 37 °C. The resulting fragments were separated by gel electrophoresis on 3% agarose gels in 1× TAE running buffer for 60 min at 100 V, stained with ethidium bromide (Sigma-Aldrich, USA), and visualized under UV light. The size of the restriction fragments was estimated using a DNA size marker (G&P Life Science, Korea).

Direct Sequencing of PCR Products. The PCR products were treated with ExoSAP-IT (USB Corporation, USA) for 15 min at 37 °C, followed by incubation at 80 °C for 15 min. One microliter of reaction mix was subjected to sequencing reactions performed in a total volume of 10 μL with 0.5 μM primer (5′-ACC TGT CTG TCA AGT CCA GCC T-3′ [forward] and 5′-TCT TCT AAG GTC CAG TCT CTG A-3′ [reverse]) using a Bigdye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) according to the manufacturer's protocols. Purification of the sequencing reactions was performed using Magnesil (Promega, USA) in 25 μL, and sequencing runs were performed on an ABI 3730 DNA analyzer (Applied Biosystems, USA). The results were analyzed by a CLC Main workbench 5.0.2 (CLC bio, Denmark).

RESULTS AND DISCUSSION

In contrast to mitochondrial DNA-based markers, no single nuclear DNA marker alone has proven to be discriminative in diverse animal species. Therefore, we sought to develop a simple nuclear DNA-based method for species identification of vertebrates. In this study, we evaluated the possible use of *ZNF238* as a successful nuclear DNA marker that can be applied to most euteleostomi for species identification. We examined the polymorphisms of *ZNF238* from as many vertebrate species ($n = 20$) as we could to evaluate the marker applicability of *ZNF238* against a wide variety of vertebrates, including major domestic animals and birds.

***ZNF238* Was Identified as a Candidate Species-Specific Marker Gene from the Bioinformatical Analysis of Single Exonic Genes.** The analysis of large numbers of genes from multiple species to identify polymorphisms and their subsequent confirmation through either genotyping or sequencing require extensive effort. Use of single exonic or intronless genes could minimize the effort in designing PCR primers for gene amplification. They could also minimize the effort in comparing sequence variations for genes with species-variable exonic structures or lengths.

To expedite the screening process of candidate genes for identifying species-specific genetic markers in vertebrates, we retrieved the DNA sequences of 3750 single exonic genes listed in the SEGE database (*18*), a database for intronless or single exonic genes in eukaryotes. Next, nucleotide sequences were bioinformatically analyzed using NCBI blast and a multiple sequence alignment program. This was done to find genes with sequence information available from several vertebrate species and well-conserved regions containing possible species-specific SNPs. From our analysis, only *ZNF238* met the criteria (data not shown). However, from further analysis on *ZNF238*, we found that *ZNF238* consisted of two exons, one large exon for the entire protein coding region and one small exon for part of the 5′ untranslated region, suggesting that the bioinformatic prediction of gene structures on a genome scale is challenging.

Determination of *ZNF238* DNA Sequences from 15 Previously Unreported Species. Because the depth of the existing sequence information for *ZNF238* in the database was shallow, we designed a specific primer set to amplify the conserved regions of *ZNF238* to obtain more sequence information from other species. The nucleotide alignment of five different species in the NCBI database allowed us to identify highly conserved regions to design PCR primers. The PCR reaction yielded the expected 823 bp products from 20 different species. PCR products from 2 to 13 individuals per species depending on the availability of samples were subjected to DNA sequence determination. The results were identical to the previously reported sequences in Genbank, except for previously unreported sequences of 15 species, *N. procyonoides*, *S. scrofa*, *L. brachyurus*, *H. inermis*, *C. hircus*, *C. capreolus*, *C. taiouanus*, *O. aries*, *M. rufus*, *A. platyrhynchos*, *P. colchicus*, *A. monachus*, *F. japonensis*, *L. africana*, and *H. amphibious* (Supporting Information), which were deposited into Genbank with accession numbers FJ799906−FJ799909, GU045454−GU045465, and GU082393−GU082394. Interestingly, the DNA sequence information from wild boars (*Sus scrofa coreanus*) and wolves (*Canis lupus chanco*) was identical to that of subspecies pigs (*Sus scrofa domesticus*) and dogs (*Canis lupus familiaris*), respectively.

In general, the usefulness of DNA markers in forensic science, food safety, and investigation of illegal wildlife traffic requires the successful analysis of the markers in low-copy and low-quality DNA samples. Therefore, further methodological adjustments for the use of *ZNF238* may be necessary to increase the usefulness of this marker in applications with field samples.

Species Discrimination by PCR-RFLP Using *Hha*I and *HpyCH4*IV. To evaluate the presence of species-specific polymorphisms of *ZNF238* and to identify possible restriction enzyme sites for PCR-RFLP, we performed in silico restriction mapping analysis for the 823 bp region of *ZNF238* against all possible restriction enzyme sites. To confirm the in silico results and to evaluate the possible presence of intraspecies variation, we performed PCR-RFLP analysis on 10 to two unrelated animals for each species. When the PCR amplicons were digested with *Hha*I restriction enzyme, eight RFLP patterns were distinctly discriminated (**Table 1**; **Figure 1**). *Hha*I generated the restriction patterns of type a in *C. familiaris* and *N. procyonoides*; b in *S. scrofa*; c in *M. musculus*; d in *E. caballus*; e in *L. brachyurus* and *L. africana*; f in *B. taurus*, *H. inermis*, *C. hircus*, *C. capreolus*, *C. taiouanus*, and *O. aries*; g in *M. rufus*, *A. platyrhynchos*, *A. monachus*, and *F. japonensis*; h in *G. gallus* and *P. colchicus*; and i in *H. amphibius*.

To distinguish the unidentifiable species by *Hha*I, *HpyCH4*IV was used (**Table 2**; **Figure 2**). *HpyCH4*IV yielded fragment patterns of type e in *C. familiaris*, *N. procyonoides*, and *E. caballus*; b in *M. musculus*; c in *L. brachyurus*; a in *S. scrofa*, *B. taurus*,

Table 1. *Hha*I Restriction Patterns Predicted for the Nucleotide Sequence of the *ZNF238* Conserved Region for 20 Vertebrate Species

RFLP type	*Hha*I restriction patterns (bp)
a	249 165 126 105 97 48 33
b	310 168 105 88 81 38 33
c	559 138 88 38
d	559 105 88 38 33
e	559 138 126
f	478 105 88 81 38 33
g	823
h	697 126
i	478 186 88 38 33

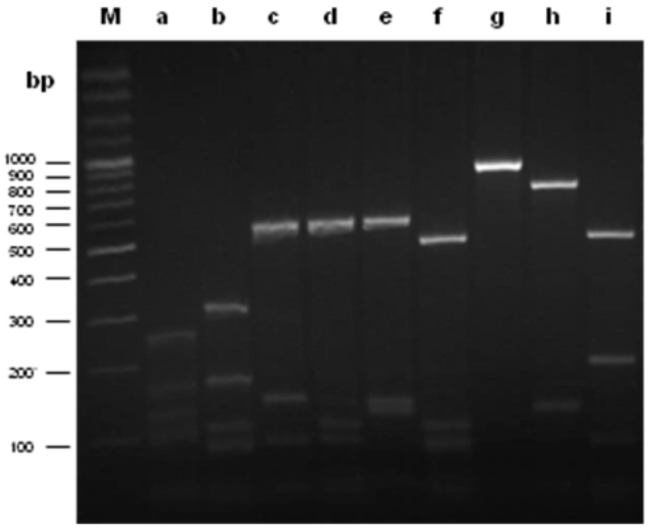

Figure 1. PCR-RFLP patterns for *ZNF238* obtained by digestion with *Hha*I. The symbols "a" to "i" on the top of the picture correspond to the PCR-RFLP types of *Hha*I in **Table 1**. DNA fragments with sizes of <100 bp were not clearly shown. M, size marker.

Table 2. *HpyCH4*IV Restriction Patterns Predicted for the Nucleotide Sequence of the *ZNF238* Conserved Region for 20 Vertebrate Species

RFLP type	*HpyCH4*IV restriction patterns (bp)
a	285 230 144 66 63 29 6
b	285 230 158 84 66
c	285 230 150 84 66 8
d	429 230 92 66 6
e	285 230 144 92 66 6
f	429 285 230 144 92 66 6
g	628 129 66
h	556 267
i	351 267 205
j	435 322 66

H. inermis, *C. hircus*, *C. capreolus*, *C. taiouanus*, and *H. amphibius*; g in *M. rufus*; h in *A. platyrhynchos*; i in *G. gallus*, *P. colchicus*, *A. monachus*, and *F. japonensis*; and j in *L. africana*. For *O. aries*, we identified a C-to-T polymorphism at cDNA nucleotide position 1101 (Supporting Information) through the process of experimental validation, resulting in three different *HpyCH4*IV RFLP patterns. The patterns of homozygotes for alleles A and B were types d and e, respectively. Heterozygotes for alleles A and B showed a banding pattern of type f (**Table 2**). We also find allelic variation for *N. procyonoides* and *F. japonensis* at nucleotide positions 1122 and 1257, respectively (Supporting Information). However, these polymorphisms were not associated with any species specificity. The results from PCR-RFLP

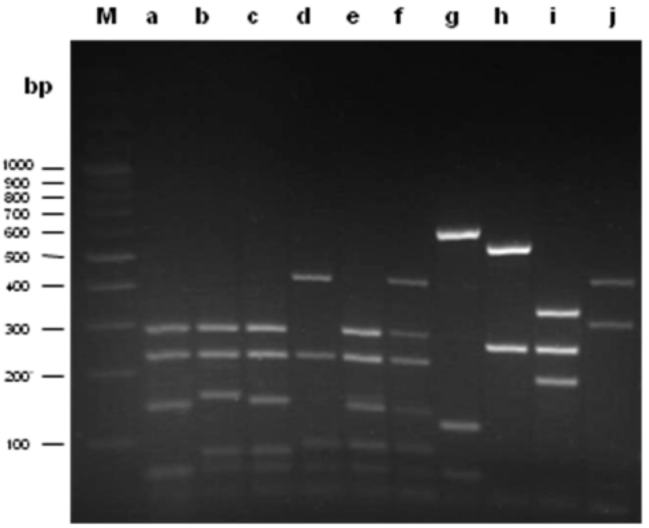

Figure 2. PCR-RFLP patterns for *ZNF238* obtained by digestion with *HpyCH4*IV. The symbols "a" to "j" on the top of the picture correspond to the PCR-RFLP types of *HpyCH4*IV in **Table 2**. DNA fragments with sizes of <100 bp were not clearly shown. M, size marker.

Table 3. *Hha*I and *HpyCH4*IV Restriction Patterns for *ZNF238* DNA Sequence Analysis for 20 Vertebrate Species

species	*Hha*I	*HpyCH4*IV
Canis lupus familiaris	a	e
Nyctereutes procyonoides	a	e
Sus scrofa	b	a
Mus musculus	c	b
Equus caballus	d	e
Lepus brachyurus	e	c
Bos taurus	f	a
Hydropotes inermis	f	a
Capra aegagrus hircus	f	a
Capreolus capreolus	f	a
Cervus nippon taiouanus	f	a
Ovis aries A	f	d
Ovis aries B	f	e
Ovis aries A/B	f	f
Macropus rufus	g	g
Anas platyrhynchos	g	h
Gallus gallus	h	i
Phasianus colchicus	h	i
Aegypius monachus	g	i
Falco peregrinus japonensis	g	i
Loxodonta africana	e	j
Hippopotamus amphibius	i	a

experiments and the predictions from in silico analyses were identical.

However, both *Hha*I and *HpyCH4*IV RFLP patterns were identical among the closely related species such as dogs and raccoons; cattle, deer (*H. inermis*, *C. capreolus*, *and C. taiouanus*) and goats; chickens and pheasants; and vultures and falcons. Therefore, we were able to distinguish 13 animal species groups, including major domestic animals, using two restriction enzymes against the conserved region of *ZNF238* (**Table 3**).

Complete Species Discrimination Using Single Nucleotide Polymoprhisms (SNPs) from the Conserved Region of *ZNF238*. To differentiate the undistinguishable species by *Hha*I and *HpyCH4*IV RFLP patterns, we analyzed SNPs for the conserved region of *ZNF238* (nt771−1549) among 21 different species, including the 20 species in this study as well as *H. sapiens* from a public database (Supporting Information). The dog and raccoon

Table 4. Differentiation of Closely Related Species Using Single Nucleotide Polymorphisms

(A)

species	nucleotide position 1032	1326
Canis lupus familiaris	C	G
Nyctereutes procyonoides	T	A

(B)

species	nucleotide position 1014	1051	1275	1440	1479
Bos taurus	A	A	G	C	T
Hydropotes inermis	C	G	G	T	C
Capreolus capreolus	C	G	G	C	C
Cervus nippon taiouanus	A	A	C	C	C
Capra aegagrus hircus	A	A	G	T	C

(C)

species	nucleotide position 1116	1167	1311
Gallus gallus	T	C	C
Phasianus colchicus	C	T	T

(D)

species	nucleotide position 921	954	1020	1455	1542
Aegypius monachus	A	T	G	G	G
Falco peregrinus japonensis	G	C	A	A	A

were distinguished by nucleotide positions 1032 and 1326 (**Table 4A**). The cattle, deer, and goat group was separated into each species on the basis of five SNPs at nucleotide positions 1014, 1051, 1275, 1440, and 1479 (**Table 4B**). Chickens and pheasants had three nucleotide differences at positions 1116, 1167, and 1311 (**Table 4C**). Vultures and falcons showed five nucleotide differences at positions 921, 954, 1020, 1455, and 1542 (**Table 4D**). Therefore, we were able to distinguish 21 vertebrate species compared in this study by analyzing the DNA sequence polymorphisms of the conserved region of *ZNF238*.

We also analyzed the average sequence similarity of the conserved region of *ZNF238* (nt771−1549) among the species used in this study. The amino acid and nucleotide sequence similarities from pairwise comparisons between humans and 20 other species for the conserved region of *ZNF238* (excluding *X. laevis*, which is distantly related) were >96.8 and >87.9%, respectively (**Table 5**). Even the amino acid sequence similarity between humans and distantly related *X. laevis* was 82.2%. These results indicate that sequence conservation of the region selected for species discrimination may be very important for *ZNF238* function. This result may suggest that the region can be applied to other euteleostomi species that were not tested in this study. Accordingly, we did not find any inconsistent DNA polymorphisms among different breeds of animals within a species or even between subspecies such as *Sus scrofa domesticus* and *Sus scrofa coreanus* and *Canis lupus familiaris* and *Canis lupus chanco*, indicating the usefulness of *ZNF238* for species discrimination (Supporting Information).

Species Identification from DNA Mixtures of Multiple Species. In addition to species identification using single species samples, we evaluated species determination from DNA mixtures of multiple species at different concentrations. DNA of *G. gallus*, *E. caballus*, and *B. taurus* was combined at ratios of 1:1:1, 2:1:1, 10:1:1, and 50:1:1, and the accuracy of species determination was

Table 5. Amino Acid and Nucleotide Sequence Similarities for the Conserved Regions of *ZNF238* Used for Species Discrimination between Humans and Other Species[a]

species	accession no.	ID (aa, %)	ID (nt, %)
Mus musculus	NM_001012330.1	99.6	95.4
Lepus brachyurus	GU045456	99.2	96.8
Bos taurus	NM_001078016.1	98.8	95
Ovis aries	FJ799908	98.8	95
Cervus nippon taiouanus	GU045460	98.8	95
Capra aegagrus hircus	GU045458	98.8	95
Hippopotamus amphibius	GU082394	98.8	95
Equus caballus	XM_001492205.2	98.1	95.6
Capreolus capreolus	GU045459	98.1	94.9
Hydropotes inermis	GU045457	98.1	94.7
Sus scrofa	GU045465	97.7	94.9
Loxodonta africana	GU082393	97.7	93.8
Macropus rufus	FJ799907	96.9	88.5
Aegypius monachus	GU045462	96.9	88.3
Falco peregrinus japonensis	GU045463−4	96.9	87.9
Gallus gallus	XM_426137.2	96.5	88.6
Phasianus colchicus	GU045461	96.5	88.3
Anas platyrhynchos	FJ799906	96.5	88.2
Canis lupus familiaris	XM_547497.2	95.8	93.5
Nyctereutes procyonoides	GU045454−5	95.8	93.5
Xenopus laevis	BC048019.1	82.2	75.8

[a] The region from amino acid 258 to 516 (nucleotides 771−1549, the numbering starts from the translation start codon of NM_205768.2) of the conserved region of ZNF238 was used for comparisons after removal of the sequences included in primers.

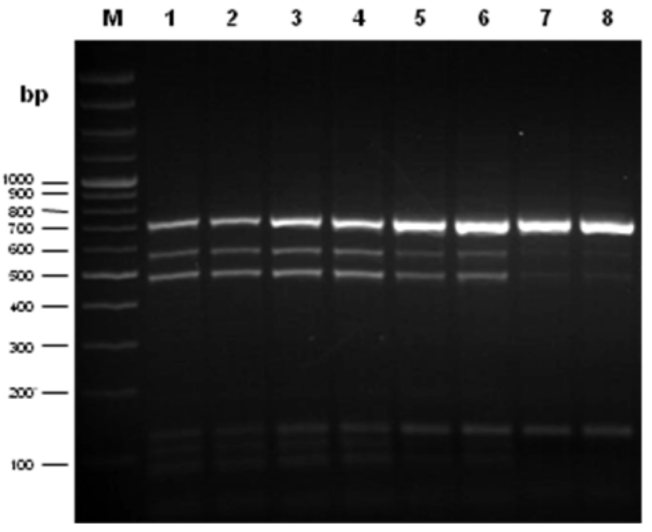

Figure 3. PCR-RFLP patterns of DNA from multiple species (*G. gallus*, *B. taurus*, *E. caballus*) with various amounts of DNA: lanes 1 and 2, 1:1:1; lanes 3 and 4, 2:1:1; lanes 5 and 6, 10:1:1; lanes 7 and 8, 50:1:1. DNA fragments with sizes of <100 bp were not clearly shown. M, size marker.

evaluated by both PCR-RFLP and direct sequencing methods. The band intensity for *G. gallus* was slightly stronger than that of other species, possibly due to a higher PCR amplification efficiency. We identified DNA banding patterns representing each species on the gel after PCR-RFLP, even at the 50:1:1 ratio (**Figure 3**).

We also attempted direct sequencing of the PCR products to more informatively present the species identification results using the same DNA mixtures. However, these results were clear only at the 10:1:1 ratio in multiple species DNA. DNA peaks from species at <10% of the total DNA amount (such as at 50:1:1) were not clearly identifiable (**Figure 4**). Although direct

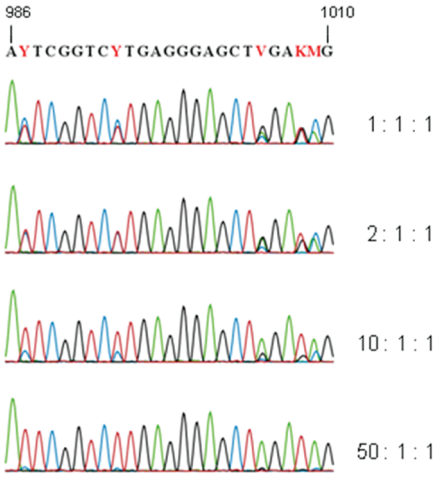

Figure 4. Sequencing chromatogram of the *ZNF238* conserved region from DNA mixtures consisting of three species (*G. gallus*, *B. taurus*, *E. caballus*) with various amounts of DNA. The total amount of template DNA per each reaction was about 50 ng. Numbers at the top of the figure indicate nucleotide positions in cDNA. Polymorphic regions have either double or triple peaks. The nucleotide position 1005 distinguishes all three species.

sequencing-based species identification using *ZNF238* was affected more significantly by differences in DNA concentration in the case of mixed DNA, it may be the more accurate method for interpreting experimental results, considering the presence of several species-specific SNPs that do not form any restriction sites (Supporting Information).

In this paper, we developed PCR-RFLP and direct sequencing methods for species identification of bony vertebrates using a new nuclear DNA marker, *ZNF238*. We showed that the conserved region of the gene can be used specifically as a marker for species identification. Because we only experimentally tested 20 species plus 2 subspecies for multiple animals, additional species should be experimentally evaluated to expand the usability of *ZNF238* as a simple and efficient nuclear DNA marker for species identification. The use of *ZNF238* together with mitochondrial analysis can provide more information and higher accuracy in species identification than relying on the results using mtDNA alone.

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Supporting Information Available: Result of multiple sequence alignment for the *ZNF238* conserved region among 21 species plus 2 subspecies of animals. The human sequence was used as a reference sequence. Nucleotides identical with the reference are indicated with a dot. Numbers indicate the nucleotide positions in cDNA (NM_205768.2). Two alleles were identified in *Nyctereutes procyonoides*, *Ovis aries*, and *Falco peregrinus japonensis*. This material is available free of charge via the Internet at http://pubs.acs.org.

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