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Species identification of animal cells by nested PCR targeted to mitochondrial DNA

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Abstract We developed a highly sensitive and convenient method of nested polymerase chain reaction (PCR) targeted to mitochondrial deoxyribonucleic acid (DNA) to identify animal species quickly in cultured cells. Fourteen vertebrate species, including human, cynomolgus monkey, African green monkey, mouse, rat, Syrian hamster, Chinese hamster, guinea pig, rabbit, dog, cat, cow, pig, and chicken, could be distinguished from each other by nested PCR. The first PCR amplifies mitochondrial DNA fragments with a universal primer pair complementary to the conserved regions of 14 species, and the second PCR amplifies the DNA fragments with species-specific primer pairs from the first products. The species-specific primer pairs were designed to easily distinguish 14 species from each other under standard agarose gel electrophoresis. We further developed the multiplex PCR using a mixture of seven species-specific primer pairs for two groups of animals. One was comprised of human, mouse, rat, cat, pig, cow, and rabbit, and the other was comprised of African green monkey, cynomolgus monkey, Syrian hamster, Chinese hamster, guinea pig, dog, and chicken. The sensitivity of the PCR assay was at least 100 pg DNA/reaction, which was sufficient for the detection of each species of DNA. Furthermore, the nested PCR method was able to identify the species in the interspecies mixture of DNA. Thus, the method developed in this study will provide a useful tool for the authentication of animal species.

Keywords Cell line authentication · Cross-contamination · Quality control · Bio-resources

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Introduction

It has been occasionally reported that cell lines derived from a certain source can be contaminated with another cell line. This cross-culture contamination is a serious problem for investigations using culture cells (Nelson-Rees et al. 1981). Therefore, it is very important to confirm the identities of cell lines as part of quality control in the operation of the cell banks that supply these cells to researchers. Some methods have been developed for the authentication of cell lines. For example, short tandem repeat profiling has been used to identify human-origin cell lines (Tanabe et al. 1999; Masters et al. 2001). As for the methods to detect interspecies cross-contamination, chromosome typing, immunological testing, and isoenzyme analysis have been used (Montes de Oca et al. 1969; Stulberg 1973; Doyle et al. 1990). Each of these methods, however, has disadvantages, such as chromosome analysis, which requires great skill, and immunological identification, which requires species-specific antibodies. Isoenzyme analysis is a general method to find

interspecies cross-contamination (Steube et al. 1995). However, the sensitivity of this technique is not suitable for the detection of intermingling with other species-derived cells (Nims et al. 1998), and some specialized reagents and devices are required.

The identification of species by polymerase chain reaction (PCR) based on species-specific deoxyribonucleic acid (DNA) sequences has many advantages, as follows: (1) the equipment required for PCR has become widespread in the laboratories of life science research, (2) the method is relatively simple and does not require great skill, and (3) the sensitivity is high because of amplification of a specific DNA fragment. Thus, some PCR methods for identification of animal species, including cell line authentication, have been reported in recent years (Naito et al. 1992; Hershfield et al. 1994; Parodi et al. 2002; Liu et al. 2003; Steube et al. 2003). However, these methods are not suitable for the purpose of rapidly distinguishing many kinds of animal species.

In the present study, we developed a highly sensitive PCR method that can distinguish 14 animal species, which are commonly used in cell cultures for life science research; i.e., human, cynomolgus monkey, African green monkey, mouse, rat, Syrian hamster, Chinese hamster, guinea pig, rabbit, dog, cat, cow, pig, and chicken.

Materials and Methods

Cell lines and preparation of DNA. All cell lines used in this study are shown in Table 1 and are available from the Health Science Research Resources Bank (HSRRB). These cell lines were confirmed to be free of microorganisms, such as mycoplasma, bacteria, fungi and yeast, and the species in the original description was authenticated by isoenzyme analysis at the HSRRB. Cellular DNA containing both nuclear and mitochondrial DNA was extracted using MagExtractor-Genome (Toyobo. Co., Ltd., Osaka, Japan) according to the manufacturer's instruction, and the resultant purified DNA was used for PCR.

Primer design. The information of full-length and partial mitochondrial DNA sequences for 14 species of animals were obtained from the published database at the National Center for Biotechnology Information (NCBI). The accession numbers of the reference sequences and the area corresponding to each primer's target are listed in Table 2, and the nucleotide sequences of each primer are presented in Table 3. The first primers, which were complementary to conserved sequences within cytochrome *b* (for forward primer) and 16S ribosomal RNA genes (for reverse) among the 14 species, were designed as a universal primer pair (Fig. 1). The amplified product covers cytochrome *b*, d-loop, 12S ribosomal RNA and 16S ribosomal RNA genes, and the predicted product size is 4–5 kbp. The species-specific sequences within the area amplified by the universal primer pair were selected as second primer pairs. To clearly identify the species-specific bands in agarose gel electrophoresis, we designed 2nd primers for the 14 species to amplify different sizes of DNA in the range of 200–1400 bp at approximately 50-bp intervals (Table 2; see also Fig. 3*A*).

Polymerase chain reaction. The 50-µl reaction mixture contained 1.25 units Takara Ex Taq (Takara Bio, Inc., Otsu, Japan), Ex Taq buffer (Mg^{2+}: 2 mM), dNTPs (50 µM each), 10 pmol of each primer and 100 ng of sample DNA, unless otherwise stated. The amplification was carried out in a PCR Thermal Cycler MP (TP3000; Takara Bio Inc.). In the first PCR, the reaction mixture was heated at 94° C for 5 min, at 59° C for 5 min, followed by 35 cycles of elongation at 72° C for 2.5 min, denaturation at 94° C for 30 s, annealing at 59° C for 45 s, with elongation at 72° C

Table 1. Cell lines used in this study

Name of cell line	Registry number	Species
293	JCRB9068	Human
A549	JCRB0076	Human
COLO320 DM	JCRB0225	Human
HuH-7	JCRB0403	Human
HeLa S3	JCRB9010	Human
Hep G2	JCRB1054	Human
JTC-12	JCRB0607	Cynomolgus monkey
MK.P3	JCRB0607.1	Cynomolgus monkey
COS-7	JCRB9127	African green monkey
Vero	JCRB9013	African green monkey
3T3-L1	JCRB9014	Mouse
A9	JCRB0221	Mouse
B16 melanoma	JCRB0202	Mouse
KUM3	JCRB1134	Mouse
WEHI-3b	IFO50296	Mouse
C6	IFO50110	Rat
L6	JCRB9081	Rat
Py-3Y1-S2	JCRB0736	Rat
WB-F344	JCRB0193	Rat
BHK(C-13)	JCRB9020	Syrian hamster
RPMI 1846	JCRB9087	Syrian hamster
CHO-K1	IFO50414	Chinese hamster
TG-1	JCRB0626	Chinese hamster
104C1	JCRB9036	Guinea pig
SIRC	IFO50020	Rabbit
MDCK	IFO50071	Dog
CRFK	JCRB9035	Cat
PG4(S+L−)	JCRB9125	Cat
MDBK	JCRB9028	Cow
PK(15)	JCRB9030	Pig
DT40	JCRB9130	Chicken
LMH	JCRB0237	Chicken
4G12 hybridoma	IFO50090	Hybrid (human × mouse)
N18-RE-105	IFO50221	Hybrid (mouse × rat)

Table 2. The target sequence position for each primer pair in the mitochondrial genome and the predicted size of the amplified product

Species	Primer						Reference mitochondrial DNA sequence (NCBI accession number)
	First primer		Second primer				
	Forward	Reverse	Forward	Reverse	Genes amplified	Predicted product size (bp)	
Human	15226–15249	2990–3009	15311–15334	15732–15751	Cyt b	441	NC 001807
Cynomolgus monkey	479–502[(a)]	1572–1591[(b)]	209–229[(c)]	1320–1340[(c)]	12S→16S	1132	(a)AF295584, (b)AF420036, (c)AF424970
African green monkey	14643–14666	2408–2427	800–823	1074–1100	12S→16S	301	AY863426.1
Mouse	14623–14646	2430–2449	28–55	954–975	tRNA-Phe→12S	948	NC 005089
Rat	14602–14625	2419–2438	1748–1767	2218–2240	16S	493	NC 001665
Syrian hamster	479–502	ND[a]	682–703	906–926	Cyt b	245	AF119265
Chinese hamster	14604–14627	2413–2432	353–376	930–953	12S	601	DQ390542
Guinea pig	14642–14665	2494–2413	140–159	454–478	12S	339	NC 000884
Rabbit	14653–14676	2425–2444	116–136	799–819	12S	704	NC 001913
Dog	14668–14691	2428–2447	1105–1125	1838–1859	16S	755	AY729880
Cat	15516–15539	3288–3307	1675–1694	3046–3065	12S→16S	1391	NC 001700
Cow	14991–15014	2781–2800	401–421	1469–1490	tRNA-Phe→16S	1090	AB074965
Pig	15791–15814	3568–3587	2099–2123	2898–2917	12S→16S	819	AY337045
Chicken	15383–15406	3715–3734	3395–3415	3570–3591	16S	197	AB086102

Cyt b cytochrome b, *tRNA-Phe* phenylalanine transfer RNA, *12S* 12S ribosomal RNA, and *16S* 16S ribosomal RNA
[a] The corresponding 16S ribosomal RNA genome sequence of Syrian hamster was not available.
[(a)] means reference sequence AF295584.
[(b)] means reference sequence AF420036.
[(c)] means reference sequence AF424970.

for 10 min in the last cycle, and stored at 4° C. The first amplified product was diluted to 1:10 with sterile distilled water, and a 1 μl aliquot of the diluted product was used as the sample DNA for the second PCR. In the second PCR, the reaction mixture was heated to 94° C for 5 min, maintained at 60° C for 5 min, followed by 30 cycles of elongation at 72° C for 1.5 min, denaturation at 94° C for 45 s, annealing at 60° C for 30 s, with elongation at 72° C for 10 min in the last cycle, and stored at 4° C. Each 5 μl of second PCR product was run on a 2% agarose (SeaKem GTG agarose; Cambrex Bio Science Rockland, Inc., Rockland, ME) minigel unless otherwise noted, stained

Table 3. Nucleotide sequences of each primer pair

Primer pair	Forward sequence	Reverse sequence
First PCR primer	THGTHSAATGAATCTGAGGVGGVT	CGATGTTGGATCAGGACATC
Second PCR primer		
Human	TATTGCAGCCCTAGCAGCACTCCA	AGAATGAGGAGGTCTGCGGC
Cynomolgus monkey	AGTGAGCGCAAACGCCACTGC	GTTAACAGTGAAGGTGGCATG
African green monkey	CCAGAAGACCCACGATAACTCTCA	TGTTAGCTCAAGGTAATCGAGTTGTAC
Mouse	GCACTGAAAATGCTTAGATGGATAATTG	CCTCTCATAAACGGATGTCTAG
Rat	CAATCCACCAAGCACAAGTG	CCCCAACCGAAATTTGGTAGTTC
Syrian hamster	GACCTCTTAGGTGTATTCCTAC	GTATGAAGAAGGGGTAGAGCA
Chinese hamster	CCGGCGTAAAACGTGTTATAGACT	GTATTAGGTATAATATCGGCAGTC
Guinea pig	GCCCTATGTACCACACTCAG	CCTTAGCTTTCGTGTGTCGGACTTA
Rabbit	CATGCAAGACTCCTCACGCCA	GGGCTTTCGTATATTCTGAAG
Dog	GCCCAACTAACCCCAAACTTA	GGTTAACAATGGGGTGGATAAG
Cat	TAGAACACCCACGAAGATCC	CATATGGTCTCTTTGGGTCG
Cow	CCTAGATGAGTCTCCCAACTC	GTTGTTTAGTCGAGAGGGTATC
Pig	CCTATATTCAATTACACAACCATGC	GCGTGTGCGAGGAGAAAGGC
Chicken	GTATTCCCGTGCAAAAACGAG	CTTAGTGAAGAGTTGTGGTCTG

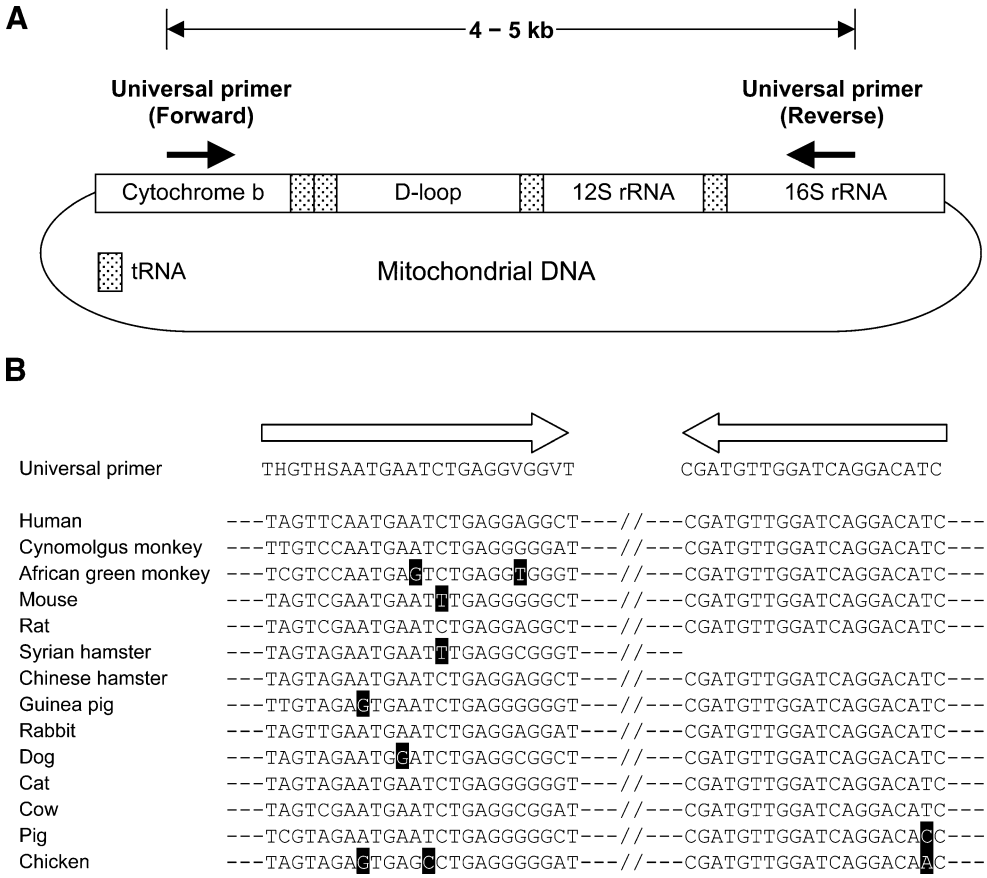

Figure 1. Universal primer pairs for the first PCR. (*A*) The target position in the mitochondrial DNA. The first PCR is expected to amplify 4- to 5-kb DNA fragments spanning from cytochrome *b* to 16S rRNA. (*B*) The sequences of the universal primers and the target nucleotide of 14 animal species. The forward primer was designed to be complementary to the conserved sequences within cytochrome *b* and the reverse primer within 16S ribosomal RNA, respectively. Degenerate primer was used for the forward primer, i.e., H;A/C/T, S;C/G, V;A/C/G. Inversed letters indicate bases mismatched to universal primer sequences. The 16S rRNA sequence of Syrian hamster for reverse primer was not available from the NCBI database.

with ethidium bromide, visualized under UV light (MupidScope WD; Advance Co., Ltd., Tokyo, Japan), and photographed. The 100 bp DNA Ladder (Takara Bio Inc.) was applied as a size marker.

Result and Discussion

First PCR. Mitochondrial DNA is generally a desirable target for PCR compared with nuclear DNA, as each animal cell generally contains 500–1,000 copies of mitochondrial DNA. Primers were designed as described in "Materials and Methods". Figure 2 shows the gel electrophoresis of the first PCR products amplified with the universal primer pair from each species DNA. The predicted 4- to 5-kbp products were clearly observed for all species, except for chicken. In the case of chicken, no visible band was observed at ca. 5 kbp, the size predicted from chicken mitochondrial DNA sequence. However, it is likely that specific amplification does occur during the first PCR for chicken DNA, because a much larger amount of chicken DNA was required without first PCR for identification during the second PCR compared with that obtained when first PCR was carried out (data not shown).

Species-specificity of nested PCR. The nested PCR strategy was used to specifically amplify species-specific DNA. To confirm amplification by each species-specific primer pair, DNA prepared from each cell line originating from 14 species of animals was subjected to the nested PCR using the universal primer pair in the first PCR and the respective single species-specific primer pair in the second PCR. The amplified product from the corresponding species DNA exhibited the predicted size (Table 2) for each animal species, and could be readily distinguished from each other according to the different sizes (Fig. 3*A*). Figure 3*B* shows the species-specificity of nested PCR in this strategy. Most of the species-specific primer pairs, i.e., human, cynomolgus monkey, Syrian hamster, Chinese ham-

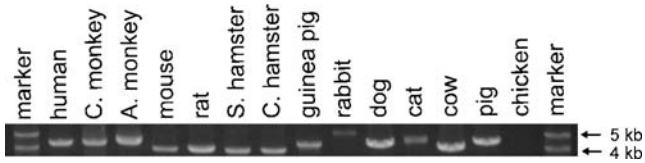

Figure 2. Gel electrophoresis of first-PCR products for 14 species. DNA of each species was extracted from the following cell lines indicated in *parentheses*, human (A549), cynomolgus monkey (MK.P3), African green monkey (COS-7), mouse (WEHI-3b), rat (Py-3Y1-S2), Syrian hamster (BHK-1 (C-13)), Chinese hamster (CHO-K1), guinea pig (104C1), rabbit (SIRC), dog (MDCK), cat (PG-4(S+L−)), cow (MDBK), pig (PK15), chicken (LMH) for amplification using the universal primer pair. The amplified DNA fragments were run on a 1% agarose gel.

ster, guinea pig, dog, cat, cow, pig, and chicken primers, amplified the specific DNA only from the corresponding species DNA. In the PCR using primer pairs specific for rabbit and African green monkey, however, unexpected bands appeared in addition to the predicted ones. The rabbit primer pair amplified cynomolgus monkey DNA, but the product could be readily distinguished from the rabbit-specific band because of their different sizes. The primer pair for African green monkey also produced an approximately 300-bp-sized band for cynomolgus monkey DNA, which was similar in size

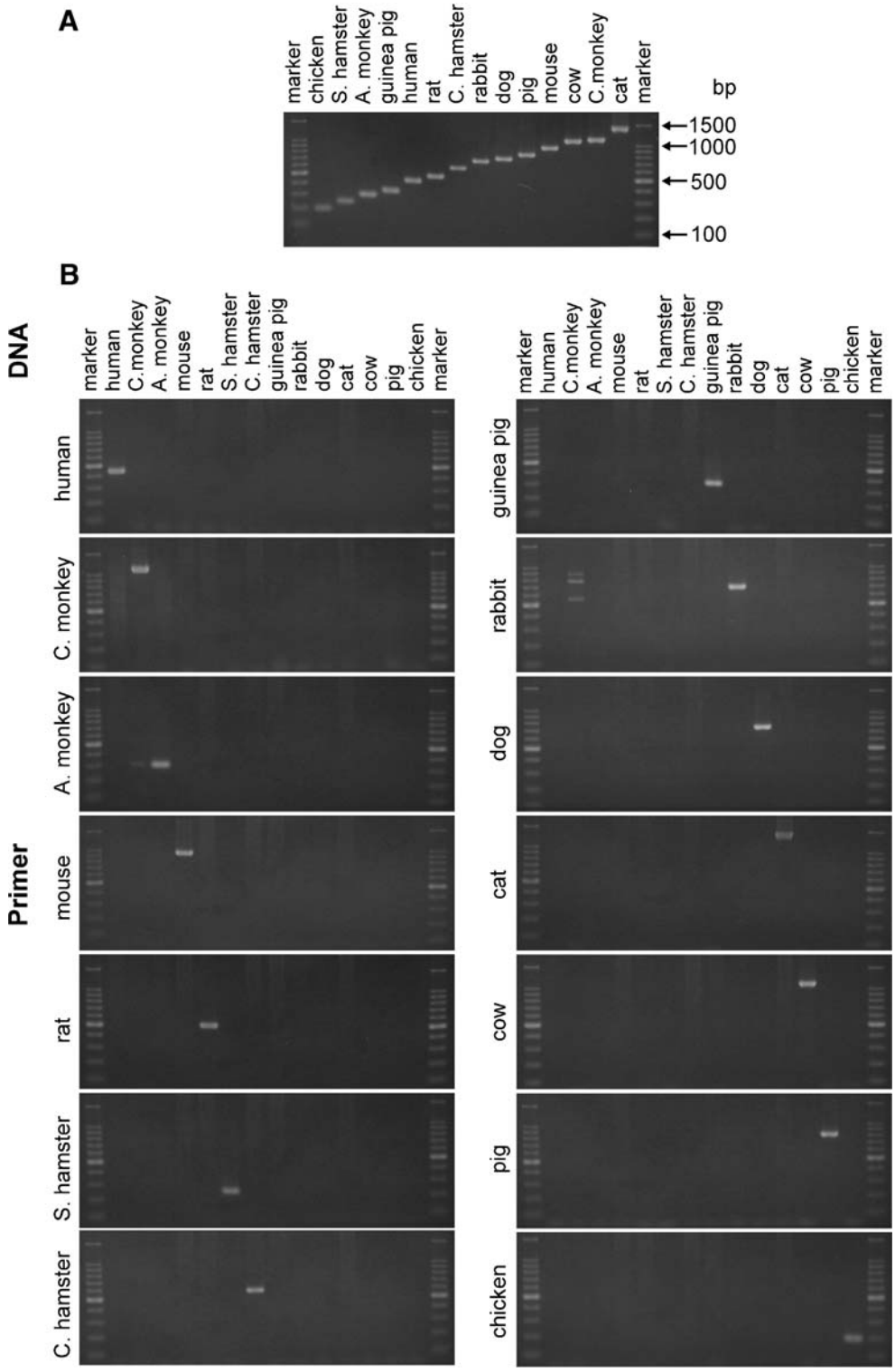

Figure 3. Gel electrophoresis of the second-PCR products for 14 species. The same cell lines as in Fig. 2 were used. (*A*) DNA of each species was subjected to nested PCR using corresponding species-specific primer pairs in the second PCR. The second-PCR products were aligned in size-order as a ladder. The amplification products were distinguished by the size. (*B*) Species specificity of the nested PCR. The 14 species-derived DNA was amplified with the universal primer pair and further amplified with the second primer pair indicated on the left side of each photograph.

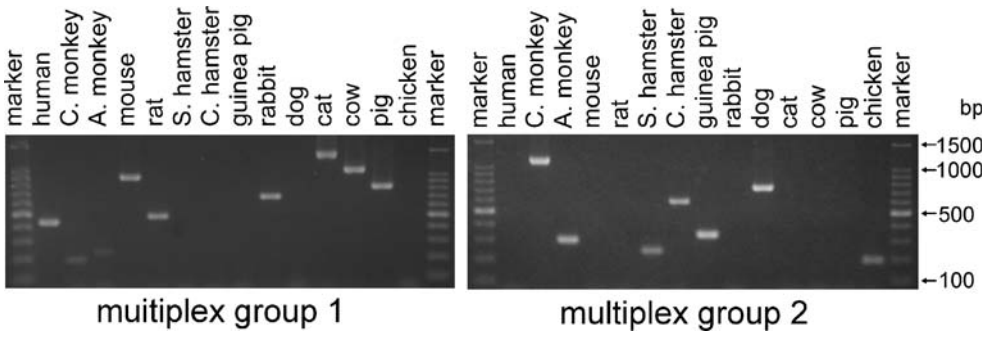

Figure 4. Gel electrophoresis of multiplex-PCR products. The first amplification products for 14 species DNA were subjected to multiplex PCR using the mixture of seven species-specific primer pairs as follows. Multiplex group 1: the primer mixture for human, mouse, rat, rabbit, cat, cow, and pig. Multiplex group 2: the primer mixture for cynomolgus monkey, African green monkey, Syrian hamster, Chinese hamster, guinea pig, dog, and chicken. The cell lines used for each animal are the same as described in Fig. 2.

to the African green monkey-specific product. This may be caused by some degree of sequence similarity between African green monkey and cynomolgus monkey in the target mitochondrial DNA. Indeed, when the mixture of primer pairs for African green monkey and cynomolgus monkey were applied to the second PCR, the nonspecific amplified product from cynomolgus monkey DNA disappeared, possibly because of competition of primer annealing to the target DNA sequences (data not shown; *see* also the result in the multiplex PCR section). Thus, it was confirmed that the nested PCR strategy is very useful for the identification of 14 species of DNA.

Multiplex PCR assay. For the simple and rapid identification of 14 species of animals, multiplex PCR was examined using primer mixtures in the second PCR. As a result of testing many combinations, it was favorable that the 14 kinds of species-specific primer pairs were divided into two groups as follows: Group 1 contained primer pairs for human, mouse, rat, rabbit, cat, cow, and pig, and Group 2 contained primers for cynomolgus monkey, African green monkey, Syrian hamster, Chinese hamster, guinea pig, dog, and chicken. Figure 4 shows the result of multiplex PCR. These animal species, divided into the two groups, could be clearly detected as species-specific bands. Most of the amplification products were specific for each primer mixture, but nonspecific bands were slightly observed for cynomolgus monkey and African green monkey when multiplex group 1 was used. These nonspecific bands were readily distinguished from the specific ones according to their sizes. Thus, it was found that multiplex PCR assay is applicable to simultaneous identification of 14 species of animals by dividing into two groups. The method developed here is superior to the previous PCR methods (Naito et al. 1992; Hershfield et al. 1994; Parodi et al. 2002; Liu et al. 2003; Steube et al. 2003) in identifying many kinds of species generally used for life science studies. In particular, this method has a great advantage in distinguishing Chinese hamster from Syrian hamster, as the cell lines such as CHO and BHK derived from these two kinds of hamsters are very popular for cell cultures.

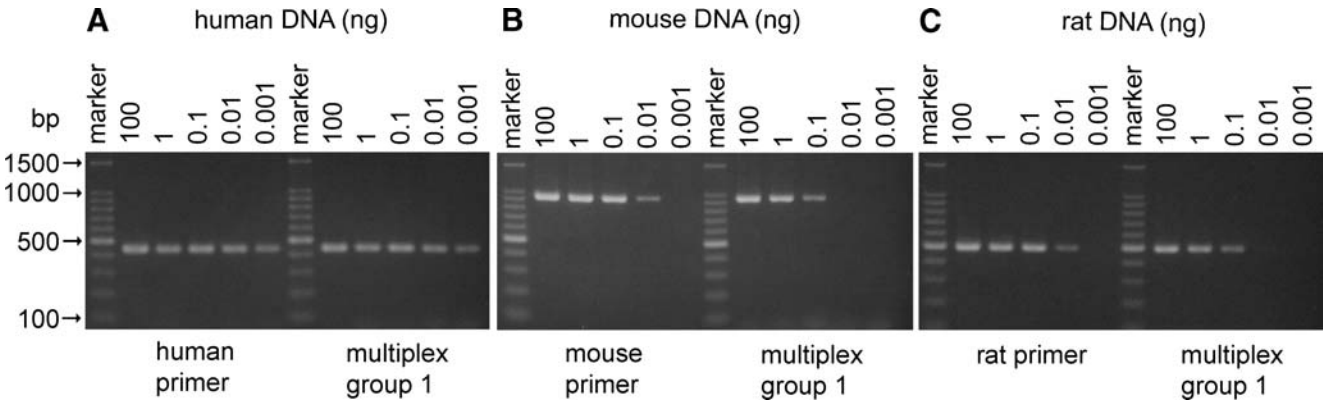

Figure 5. Gel electrophoresis of nested-PCR products for serially diluted DNA. The sample DNA was extracted from the human A549 cell line (*A*), mouse WEHI-3b cell line (*B*), and rat Py-3Y1-S2 cell line (*C*), and diluted serially to the nested PCR. The product bands amplified by single species-specific primer pairs are shown on the *left side* and those by multiplex group 1 are on the *right* in each photograph.

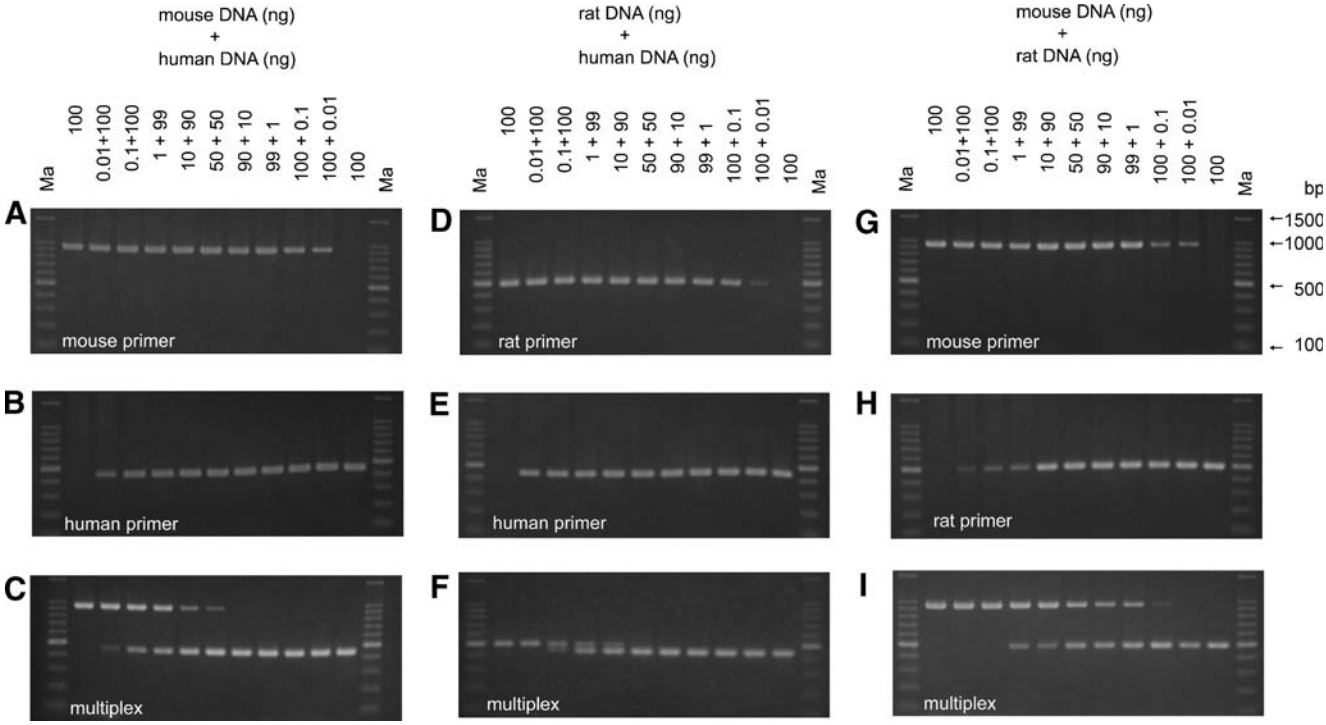

Figure 6. Gel electrophoresis of nested-PCR products for interspecies DNA mixtures. Two kinds of DNA, such as human and mouse DNA (*A*, *B*, *C*), human and rat DNA (*D*, *E*, *F*), and mouse and rat DNA (*G*, *H*, *I*) were mixed in various ratios for amplification with nested PCR. The cell lines used were the same as in Fig. 5. In the second PCR, the single species-specific primer pairs (*A*, *B*, *D*, *E*, *G*, *H*) or the multiplex group 1 (*C*, *F*, *I*) were used.

Sensitivity of PCR assay. Serially diluted cellular DNA was amplified with the nested PCR using either the corresponding species-specific primer pair or the mixture of seven species-specific primer pairs (multiplex PCR described above) as the second PCR primer. Each of the 14 species of DNA was detectable from at least 100 pg DNA/reaction by both PCR assays. Figure 5 shows the sensitivity of the PCR assay, as an example, using DNA prepared from human, mouse, and rat cell lines, which are commonly used for cell culture experiments. The amount of DNA required for identification of each species was 10 pg/reaction or more for the single species-specific primer pair as the second primer, and 100 pg/reaction or more for the multiplex assay. The sensitivity of the multiplex assay was somewhat low compared to the species-specific single primer.

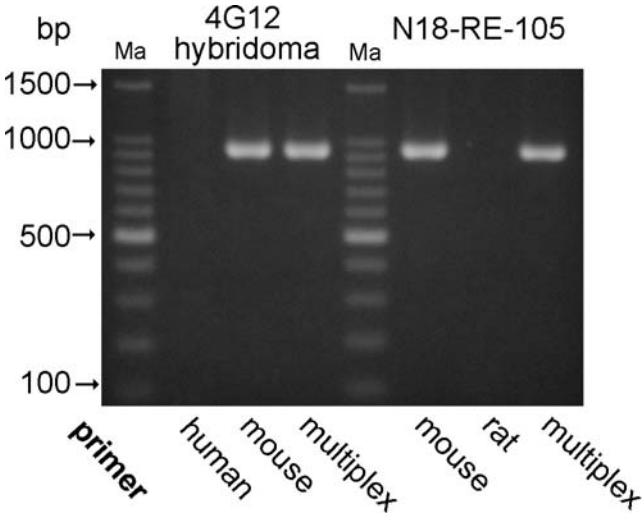

Figure 7. Gel electrophoresis of nested-PCR products for DNA derived from interspecies hybrid cell lines. DNA from 4G12 hybridoma (human × mouse) and N18-RE-105 (mouse × rat) were applied to nested PCR. Multiplex group 1 or the corresponding species-specific primer pairs were used in the second PCR.

Identification of species from interspecies DNA mixtures. The possibility of cross-contamination or replacement of cells exists during the process of cell preparation. As part of the quality control of cell lines in the cell bank, it is very important to verify the source species of each derived cell line. For that purpose, we attempted to identify the species from interspecies DNA mixtures. Two species of DNA, among human, mouse and rat, were mixed in various ratios for the nested PCR. When the single species-specific primer pair was used in the second PCR, each species of DNA was sensitively detected even when two kinds of DNA were present in the mixture. For example, when mouse-specific primers were used, a mouse-specific band was detected in the DNA mixture composed of 100 ng human or rat DNA + 10 pg mouse DNA (Fig. 6*A*, *G*). Likewise, in the case of human-specific or rat-specific primers alone, their respective species-specific band was also detected at 10 pg DNA

(Fig. 6*B, E, D, H*). When group 1 of the multiplex primers (seven species-specific primer pairs composed of human, mouse, rat, rabbit, cat, cow, and pig) was used at the standard concentration (10 pmol each species-specific primer/50-μl reaction), the sensitivity apparently decreased and there was considerable difference in the sensitivity for human, mouse, and rat DNA (Fig. 6*C, F, I*). This may be caused by the different amplification efficiency of each species-specific primer in the simultaneous reaction. Indeed, by decreasing the ratio of human primer pairs relative to the others, the sensitivity for mouse and rat DNA clearly increased (data not shown). Thus, this method will likely become a very useful tool for quickly detecting cross-contamination, and the sensitivity in the multiplex assay will be further increased by optimizing the concentration and the ratio of species-specific primers.

Hybrid cell lines. We applied this PCR method to original-species verification of interspecies hybrid cell lines. The hybrid cell lines of 4G12 (human B lymphocytes × mouse myeloma cell line; Saito et al. 1988) and N18-RE-105 (mouse glioma cell line × rat neural retina cells; Malouf et al. 1984) were tested by isoenzyme analysis and nested PCR. Although the original species were confirmed by isoenzyme analysis of both hybridomas between human and mouse, and between mouse and rat, only the mouse-specific band was observed for both hybridomas by nested PCR (Fig. 7). This result is consistent with the previous reports that the mouse mitochondria dominate selectively in these hybrid cells, whereas human or rat mitochondria are ultimately excluded from the hybrid cells (Attardi and Attardi 1972; Yamaoka et al. 2001). The nested PCR method targeted to the mitochondria genome was not applicable to the parental species identification of interspecies hybrid cells.

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