Table 1 Sequence information of primers for species-specific PCR

Target animal	Primer name	Primer sequence (5'–3')	Product length
Donkey	EaCytB-f	GCCCTTATCCTTTCCATCTTA	66 bp
(*Equus asinus*)	EaCytB-r	GCTTCGTTGTTTTGTCATG	
Horse	EcCytB-f	CCCATTCCACCCATATTAT	71 bp
(*Equus caballus*)	EcCytB-r	GAGTTAGTAGGAGCAAGATC	
Cattle	BtCytB-f	CCATCGGACAACTAGCATCTG	68 bp
(*Bos taurus*)	BtCytB-r	TGTGCCGGCCGTTGGTATTAGC	
Pig	SsCytB-f	CTAGTAGCAGACCTCATTA	76 bp
(*Sus scrofa*)	SsCytB-r	GTTGGCCGATGATGATGAAC	

The underlined nucleotide in the donkey sequence was the one that was replaced by another nucleotide to enhance the specificity

MinElute PCR purification kit (Qiagen), subcloned into a 2.1-TOPO vector (Invitrogen/Life Technologies), and sequenced. Cycle sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems/Life Technologies), and the results were analyzed using an ABI Prism 3130 genetic analyzer (Applied Biosystems/Life Technologies).

Detection sensitivity analysis of cattle DNA

We mixed 0.1 % of cattle glue with ACC and used 5 g of the mixture for DNA extraction as described above. Using the DNA as a template, PCR with cattle-specific primers was performed to investigate the detection sensitivity of cattle DNA.

Results

Examination of DNA extraction method from ACC

Lv et al. [3] compared three strategies, the "SDS/proteinase K" (phenol/chloroform) method, the "Wizard magnetic DNA purification system for food" method, and the modified silica-based method, for extracting DNA from ACC. They concluded that the modified silica-based method was the best because it achieved higher DNA yield and purity than the others. In this study, we compared the silica-based method by Lv et al. with the ion-exchange resin-based method (Qiagen Genomic tip 20/G), which was recommended for DNA extraction from genetically modified foods [16].

The DNA extracted by each method from 1 g of donkey glue differed little in purity as estimated from the $A_{260/280}$ value. Ten ng of each DNA was amplified by PCR with primers based on ERE-1 (equine SINE) in the same way as reported by Lv et al., which resulted in the generation of each PCR amplicon with the predicted size. However, more amplicons were obtained from DNA extracted by the

ion-exchange resin method than by the silica-based method, though the same amount of DNA was used in PCR (Fig. S2).

Lv et al. had reported that the SINE region was amplified, but mitochondrial regions such as cytochrome *b* were not amplified using DNA extracted by the silica-based method [4]. As the ion-exchange resin method was shown to be effective in obtaining more amplicons of the SINE region, we tried to amplify the cytochrome *b* region, and were able to obtain amplicons using 50 ng of DNA extracted by the ion-exchange resin method. These results suggest that the ion-exchange resin method is superior to the silica-based one for extraction from ACC.

As mentioned above, mitochondrial DNA sequences can distinguish donkey from horse sources, unlike those of SINE. Because it was confirmed that the cytochrome *b* region can be amplified from DNA of ACC, we selected that region as the target in species-specific PCR.

Establishment of species-specific PCR

ACC adulterated with substances from other animals such as horses, cattle, and pigs has been found in Chinese markets [3, 4]. We tried to establish PCR methods for species-specific detection of substances derived from donkey as well as horse, cattle, and pig-.

As DNA extracted from donkey glue is highly degraded, amplicons more than 100 bp long are difficult to obtain [3]. The primers were designed based on the species-specific sequences in the cytochrome *b* gene so that the size of amplicons was less than 100 bp (Table 1; Fig. S1). PCR was performed using 5 ng of DNA extracted from each animal sample with the designed species-specific primers. As a result, amplicons specific to each animal were obtained (donkey, 66 bp; horse, 71 bp; cattle, 68 bp; pig, 76 bp) (Fig. 1a–d). Sequencing analysis after subcloning of the PCR products confirmed that all of the sequences showed good identity with cytochrome *b* genes of the corresponding target animals.
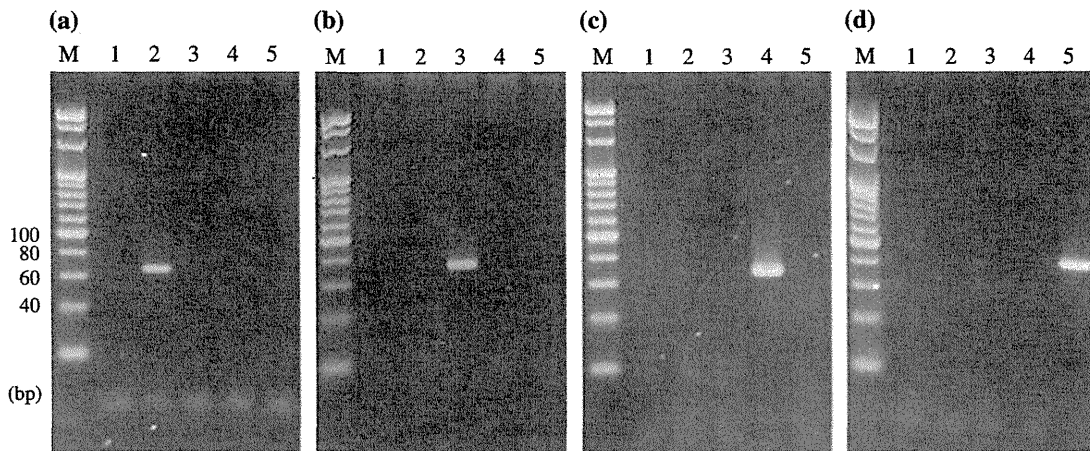
(a)

(b)

(c)

(d)

Fig. 1 Specificity of PCR for donkey, horse, cattle, and pig DNA with each of their specific primers. **a** Donkey-specific PCR. **b** Horse-specific PCR. **c** Cattle-specific PCR. **d** Pig-specific PCR. *Lane 1* is a no-template control, and *lanes 2–5* show the DNA from donkey, horse, cattle, and pig samples, respectively. *M*, 20-bp ladder marker

Fig. 2 Specificity of PCR for DNA from ACC with each of four animal-specific primers. **a** Donkey-specific PCR. *Lane 2*, positive control (PC; donkey DNA), **b** Horse-specific PCR. *Lane 2*, PC (horse DNA). **c** Cattle-specific PCR. *Lane 2*, PC (cattle DNA). **d** Pig-specific PCR. *Lane 2*, PC (pig DNA). *Lanes 1* and *3* indicate no-template control and ACC, respectively, in all panels. *M*, 20-bp ladder marker

(a)

(b)

(c)

(d)
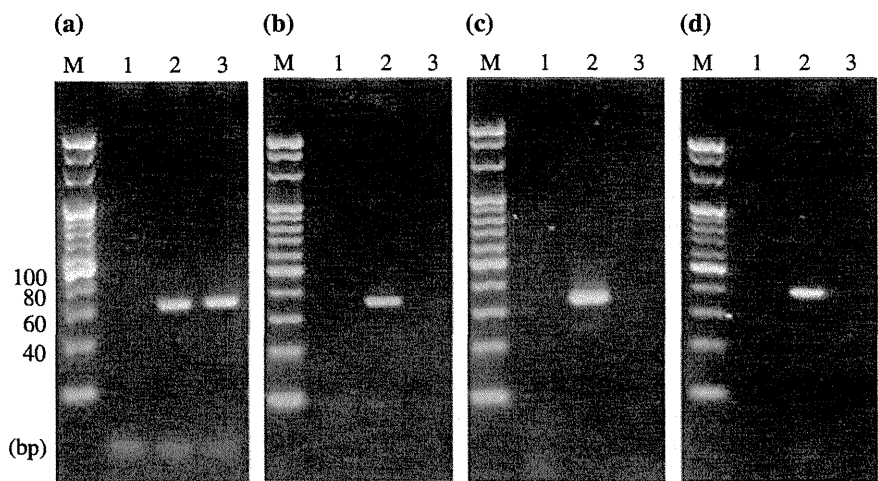
These species-specific PCR methods were applied to DNA extracted from ACC known to be derived from donkeys only. Amplicons could be obtained only from the donkey-specific PCR (Fig. 2a–d). These results suggest that the species-specific PCR methods established in this study would be useful for simple and easy identification of the animal origin of ACC.

Detection sensitivity of cattle DNA

In order to estimate the detection sensitivity of the PCR methods in this study, the detection limit was analyzed using cattle glue as a contaminant. DNA extracted from ACC blended with 0.1 % of cattle glue was applied to the cattle-specific PCR, and the cattle-specific amplicon was detected (Fig. 3). Thus, the PCR method was confirmed to
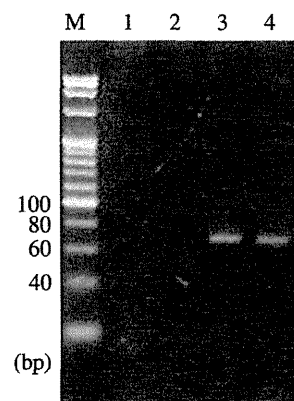

Fig. 3 Evaluation of cattle-specific PCR assay sensitivity. *Lane 1*, no-template control; *lane 2*, ACC; *lane 3*, 0.1 % cattle glue in ACC; *lane 4*, cattle glue; *M*, 20-bp ladder marker

be able to detect as little as 0.1 % admixture of cattle glue in ACC.

Discussion

In this study, new PCR methods were established to identify the animal origins of ACC. First, the silica-based and the ion-exchange resin methods were compared as methods for extracting DNA from ACC, and the ion-exchange resin method was revealed to be more suitable. This may be because the ion-exchange resin procedure was so gentle that DNA fragments were not degraded further during preparation, or was superior for removing polysaccharides and phenols that inhibit the activity of the PCR enzyme.

PCR methods based on the cytochrome *b* gene were established to detect DNA specific to donkeys, horses, cattle, and pigs. The methods were shown to be effective in identifying the animal origins of ACC. Unlike the SINE-based PCR, the cytochrome *b*-based method could directly distinguish donkey- from horse-derived substances, and should be considered as a new method for simple and easy identification of the animal origins of ACC.

The detection sensitivity of the PCR methods against ACC blended with DNA from other animal species was also investigated. Because horse glue and pig glue could not be obtained, only cattle glue was used for detection limit analysis. As a result, a 0.1 % mixture of cattle was detected in the cattle-specific PCR, which was as sensitive as in the SINE-based method and sensitive enough to examine impurity contamination. On the other hand, the cattle-specific PCR did not have quantitative capability, given the fact that there was little difference in the amount of amplicons from 0.1 and 100 % cattle glue. One of the reasons may be that we detected the amplicons at the endpoint of amplification in the PCR method. For quantitative analysis, further investigations into quantitative PCR would be needed.

References

1. The Japanese standards for non-Pharmacopoeial crude drugs (non-JP crude drug standards) (2012) Ministry of Health, Labour and Welfare, Japan
2. Pharmacopoeia of the People's Republic of China, English Edition 2010. China Medical Science Press, Beijing, China
3. Lv P, Zhou XS, You JH, Ye BC, Zhang YX (2009) Extraction of trace amount of severely degraded DNA. Z Naturforsch 64c: 581–589
4. Lv P, Zhao YJ, Qi F, Zhou XS, You JH, Qin YF, Zhang YX (2011) Authentication of equine DNA from highly processed donkey-hide glue (*Colla Corii Asini*) using SINE element. J Food Drug Anal 19:123–130
5. Xu CH, Zhou Q, Sun SQ, Wang BQ (2005) The identification of Ejiao by two dimensional correlation infrared spectroscopy. Chinese J Anal Chem 33:221–224
6. Qu HB, Yang HL, Chen YY (2006) Fast and nondestructive discrimination of donkeyhide glue by near-infrared spectroscopy. Spectrosc Spect Anal 26:60–62
7. Wang WJ, Guan Y, Zhu YY (2007) Novel identification of donkeyhide glue by X-ray fluorescence analysis. Spectrosc Spect Anal 27:1866–1868
8. Ballin NZ, Vogensen FK, Karlsson AH (2009) Species determination—can we detect and quantify meat adulteration? Meat Sci 83:165–174
9. Shedlock AM, Takahashi K, Okada N (2004) SINEs of speciation: tracking lineages with retroposons. Trends Ecol Evol 19:545–553
10. Sakagami M, Hiromura K, Chemnick LG, Ryder OA (1999) Distribution of the ERE-1 family in Perissodactyla. Mamm Genome 10:930–933
11. Pauciullo A, Kubickova S, Cernohorska H, Petrova K, Di Berardino D, Ramunno L, Rubes J (2006) Isolation and physical localization of new chromosome-specific centromeric repeats in farm animals. Vet Med 51:224–231
12. Krcmar P, Rencova E (2003) Identification of species-specific DNA in feedstuffs. J Agric Food Chem 51:7655–7658
13. Pascoal A, Prado M, Calo P, Cepeda A, Barros-Velazquez J (2005) Detection of bovine DNA in raw and heat-processed foodstuffs, commercial foods and specific risk materials by a novel specific polymerase chain reaction method. Eur Food Res Technol 220:444–450
14. Martin I, Garcia T, Fajardo V, Lopez-Calleja I, Hernandez PE, Gonzalez I, Martin R (2007) Species-specific PCR for the identification of meat species in feedstuffs. Meat Sci 75:120–127
15. Chrisholm J, Conyers C, Booth C, Lawley W, Hird H (2005) The detection of horse and donkey using real-time PCR. Meat Sci 70:727–732
16. The method for examining genetically modified foods, final revision (2008) Ministry of Health, Labour and Welfare, Japan
17. Latorra D, Campbell K, Wolter A, Hurle JM (2003) Enhanced allele-specific PCR discrimination in SNP genotyping using 3' locked nucleic acid (LNA) primers. Hum Mutat 22:79–85