Journal of Food Measurement and Characterization (2021) 15:1161–1168
https://doi.org/10.1007/s11694-020-00717-5

ORIGINAL PAPER

Multiplex and real-time PCR for qualitative and quantitative donkey meat adulteration

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Received: 16 June 2020 / Accepted: 18 October 2020 / Published online: 26 October 2020

Abstract

Donkey meat is a rich source of protein excellent in indispensable amino acid and low in fat. Its quality has received great attention due to its high nutritional and medicinal value to human beings. Due to donkey resource scarcity and gradually increasing market demand for donkey meat products, adulterated donkey meat products with other low cost animal meat, especially sheep, cattle, pig and horse, were often found in market and had raised widespread concern in recent years. In this study, total 300 mg samples of binary mixtures, containing 90%, 50%, 25%, 10%, 5%, 1% (w/w) of donkey in horse meat respectively, were used for DNA extraction. Both conventional multiplex and real-time PCR techniques were developed for qualitatively and quantitatively detecting the adulteration in donkey meat. The multiplex PCR allowed rapid and simultaneous qualitative detection of donkey and four common adulterated species including sheep, cattle, pig and horse. Moreover, a normalized real-time PCR assay was further developed to detect and quantify the donkey mitochondrial DNA, and the results of simulate adulteration suggested this method could quantitatively detect donkey meat in range of 1–90% with high trueness. The results illustrated that both methods might be applied to detect commercial foodstuff adulteration. Therefore, both improved conventional multiplex and real-time PCR methods were developed to achieve qualitative and quantitative detection of the adulteration in donkey meat products.

Keywords Conventional PCR · Real-time PCR · Authentication · Donkey meat

Introduction

Donkey meat is a rich source of protein excellent in indispensable amino acids which includes leucine, lysine, methionine, etc. Compared to other livestock animals, it also contains a high content of unsaturated fatty acids and polyunsaturated fatty acid (PUFA), exhibiting a higher nutritional value [1]. Economically motivated adulteration (EMA), including meat adulteration with low priced or low valued meat varieties, has been a common practice in both developing and developed countries [2, 3]. Meat adulteration triggers not only economic concerns, but also religious, ethical and health issues [4]. EMA also exists in donkey-derived products, which are served as raw material of the traditional Chinese medicine since ancient times [5]. Donkey-hide gelatin (*Colla corii asini*), recorded in the ancient Chinese medical document, mainly derived from the donkey skin (*Equus asinus*) by soaking and stewing. Donkey-derived gelatin promotes hematopoiesis, arrests bleeding, and also in treatment of gynecologic and other chronic ailments [6]. Nowadays, the soaring demand for donkey products and a shortage of donkey population had encouraged a boom in imitation products. Multiple available methods for species identification in raw meat and other food products such as the lipid-based techniques [7], volatile organic compounds analyses [8–10] and protein-based procedures [11–13] have been developed. Nevertheless, these methods are either time-consuming or inaccurate owning to the protein ingredient that would be damaged during thermal stress, high pressure and other processing technologies [14]. Moreover, they may not be adequate to discriminate adulteration between species that are closely related, or may

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not be suitable for processed meats. It is well-known that the DNA molecule is more stable than protein and could be successfully extracted from raw meat and other highly degraded food samples [15–17]. Accordingly, the DNA-based method is becoming a relatively reliable, rapid and economy way for the detection of meat adulteration [18]. Nowadays, few DNA based techniques have reported to qualitative and quantitative detect donkey meat, such as PCR-RFLP, conventional PCR and real-time PCR that have been established for qualitative or quantitative detection of adulteration in donkey meat [19–22]. The restriction endonucleases are essential for PCR-RFLP method, which lead to increase of the costs and labor, therefore limit its widespread use. Although conventional PCR technique could sensitively detect donkey and horse meat, however, the lack of common species such as pigs, cattle, and sheep can easily lead to false negative. Higher cost of TaqMan probe synthesis and inaccurate detection in some cases are two major shortcomings of TaqMan based real-time PCR [22]. While meat DNA denatured or some additions of vegetable with PCR inhibitor included, the proposed method is not able to reach an accuracy result [23]. Accordingly, there is no more convenient, reliable and precise method for qualitative and quantitative detection of donkey meat adulteration.

In conclusion, it is highly urgent to establish qualitative and quantitative detection method for the adulteration in donkey products. In the present study, both improved conventional multiplex and real-time PCR methods were developed to achieve qualitative and quantitative detection of the adulteration in donkey meat. The multiplex PCR technique could simultaneously, specifically and sensitively distinguish the donkey and four common potential adulterate species, including sheep, cattle, pig and horse. Moreover, SYBR green I based real-time PCR technique could quantitatively detect donkey meat with high accuracy, and could further validate with binary meat mixtures.

Materials and methods

Raw meat samples preparation

The fresh raw meats of ten species, including donkey (*Equus asinus*), horse (*Equus caballus*), pig (*Sus scrofa*), sheep (*Ovis aries*), cattle (*Bos taurus*), duck (*Anas platyrhynchos domesticus*), chicken (*Gallus gallus domesticus*), goose (*Anser cygnoides orientalis*), fox (*Vulpes*) and quail (*Coturnix*), were purchased from local market in Hangzhou China and packaged respectively in different sterile plastic bags to prevent cross-contamination. All samples were immediately stored at − 20 °C until DNA extraction.

Donkey and four potential adulterate species, including sheep, cattle, pig and horse, were finally selected to develop conventional multiplex PCR and real-time PCR assays. For simulating the adulteration problem, binary meat mixtures containing 90%, 50%, 25%, 10%, 5%, 1% (w/w) of donkey in horse meat were also prepared. Total 300 mg samples of binary mixtures were used for DNA extraction.

DNA extraction

Genomic DNA of the raw meats of aforementioned ten species as well as the simulated adulteration samples was extracted according to the manufacturer instrument of genomic DNA extraction kit (Easy-do, China). DNA concentration and purity was measured with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and all of extracted DNA was diluted with 100 uL TE buffer to final concentration of 5 ng/μL and immediately stored in − 20 °C until use.

Primer design

The species-specific PCR primers were designed according to the mitochondrial 12S rRNA sequences of each target species from GenBank. The GenBank accession numbers for these sequences were listed in Table 1. Moreover, five pairs of species-specific PCR primers and a pair of reference primer genomic 18S rRNA gene for normalize system [24] were used in this research. All the primers were listed in Table 2 and synthesized (Shangya, China).

Conventional multiplex PCR reactions

The amplifications by conventional multiplex PCR were performed in a 10 μL volume as follows: 5 μL 2×Taq Plus Master Mix(Vazyme, China), 2 μL ddH_2O, 1 μL of each species-specific primers (10 μM) and 1 μL DNA template

Table 1 NCBI Accession Numbers of 12S rRNA of five species

Species	NCBI accession numbers
Donkey	MG885769.1; MG931481.1; NC_001788.1
Sheep	KM233163.1; KP981378.1; HM236175.1; KP702285.1; MG489885.1
Cattle	KU891850.1; EU177818.1; KF926377.1; MF959941.1; AY676859.1
Pig	AF304202.1; AY574048.1; AF034253.1; AY337045.1
Horse	MG001421.1; MG001422.1; MG001428.1; MG001432.1; MG761996.1

Table 2 Primers for PCR amplifications

Species	Target gene	Primer sequences (5′→3′)	Amplicon (bp)	References
Donkey	12S rRNA	F: GAACAAGAACTCAACCCAAACGG	184	This study
		R: CTTTCATATGTTTGGATCATGGTTTTG		
Sheep	12S rRNA	F: CAAAATTATTCGCCAGAGTACTACCG	231	This study
		R: CTCCATAGGTTACACCTTGACCTAACGTC		
Cattle	12S rRNA	F: CCCTCTAGGTTGTTAAAACTAAGAGGAGCT	341	This study
		R: GTTTGGGTCTTAGCTATAGTGCGTCG		
Pig	12S rRNA	F: ACGGTAGCTCATAACGCCTTGCTC	481	This study
		R: TGAATTGGCAAGGGTTGGTAAGGTC		
Horse	12S rRNA	F: AGAATGCCCTCTAAATCGCGTCT	626	This study
		R: CCTTGACCTAACGTTTTTATGTTGGATAT		
Reference	18S rRNA	F: CTGCCCTATCAACTTTCGATGGTA	113	Costa et al. [24]
		R: TTGGATGTGGTAGCCGTTTCTCA		

(5 ng/μL). PCR amplifications were performed using a MyGene L series thermal cycler with following PCR condition: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 62.5 °C for 30 s, extension at 72 °C for 10 min. Amplified PCR products were finally separated using 2% agarose gel electrophoresis with Goldview nucleic acid stain. The agarose gel was visualized under UV light and image was captured with Tanon 2500 (Tanon, China).

Real-time PCR reactions

The real-time PCR were carried out in 10 μL volume containing 5 μL 2 SYBR Green® Premix Ex Taq™ (Takara, Japan), 3.8 μL ddH_2O, 1 μL of each primers (10 μM) and 0.8 μL DNA template. The real-time PCR program was performed by the CFX96 real-time detection system (Bio-Rad, USA) under the following conditions: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Each PCR reaction was repeated for four times, and the negative control, replacing the genomic DNA with TE buffer, was performed to monitor any possible contamination.

Result

DNA extracts

The DNA purity and concentration of the isolated DNA from each species were measured by NanoDrop 2000 spectrophotometer as well as agarose gel electrophoresis. The value of OD_{260}/OD_{280} and concentration were at the range of 1.8 to 2.0 and 15 to 25 ng/μL, respectively. Furthermore, the reference gene of each extracted DNA was PCR amplified and sequenced. The detailed sequences of the amplicons were identical to the predicted sequences, guaranteeing the authenticity of the experimental samples. Accordingly, the extracted DNA was suitable for the following assays.

Conventional PCR assay

Specificity test

Five pairs of species-specific primers were applied to amplify 12S rRNA gene from five different species (donkey, horse, pig, sheep and cattle). The results revealed that only the species-specific PCR primers could trigger the expected amplification and gain the expected PCR fragments. Detailedly, the expected lengths of species-specific fragments was 184 bp, 231 bp, 341 bp, 481 bp and 626 bp for donkey, sheep, cattle, pig and horse respectively (Fig. 1). Moreover, no cross-reactivity was found (Fig. 1). All the five species-specific PCR products were verified by sequencing.

Multiplex amplification

The multiplex amplification assay for simultaneous detection of the five meat species was successfully developed using five pairs of spices-specific primer and genomic DNA mixture of five species (listed in Table 3). As shown in Fig. 2a, the lengths of five PCR fragments were consistent with those of single amplification as expected. All the PCR fragments were purified and verified by sequencing.

Sensitivity test

The sensitivity test of conventional multiplex PCR was carried out by mixing five different genomic DNA with appropriate ratios of 0.01–20% (Fig. 2b). The mixtures with five different DNA concentrations were amplified and result showed that the sensitivities threshold was 0.1% (5 pg/μL) for donkey, sheep, cattle and pig (Fig. 2b). Meanwhile,

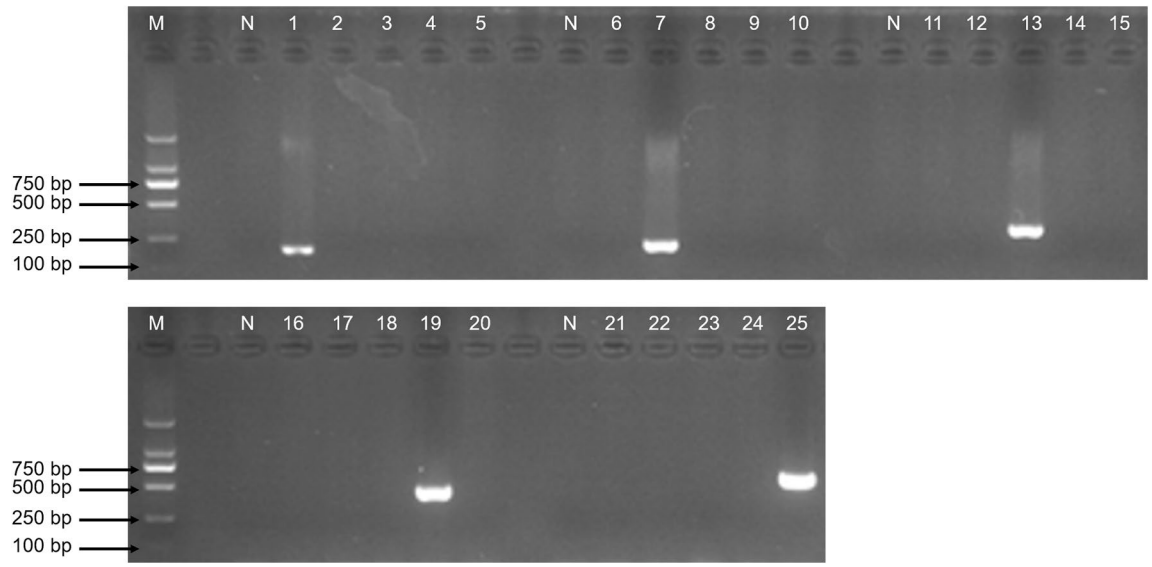

Fig. 1 Specificity test of the species-specific PCR primers. (M) DNA Marker, DL2000. Lanes 1–5: donkey, sheep, cattle, pig, horse genomic DNA with donkey specific primer. Lanes 6–10: donkey, sheep, cattle, pig, horse genomic DNA with sheep specific primer. Lanes 11–15: donkey, sheep, cattle, pig, horse genomic DNA with cattle specific primer. Lanes 16–20: donkey, sheep, cattle, pig, horse genomic DNA with pig specific primer. Lanes 21–25: donkey, sheep, cattle, pig, horse genomic DNA with horse specific primer. Lane N: negative control

Table 3 Composition of genomic DNA meat samples for sensitivity test

	Donkey	Sheep	Cattle	Pig	Horse	TE buffer
Mixture 1 (%)	20	20	20	20	20	0
Mixture 2 (%)	10	10	10	10	10	50
Mixture 3 (%)	1	1	1	1	1	95
Mixture 4 (%)	0.1	0.1	0.1	0.1	0.1	99.5
Mixture 5 (%)	0.01	0.01	0.01	0.01	0.01	99.95

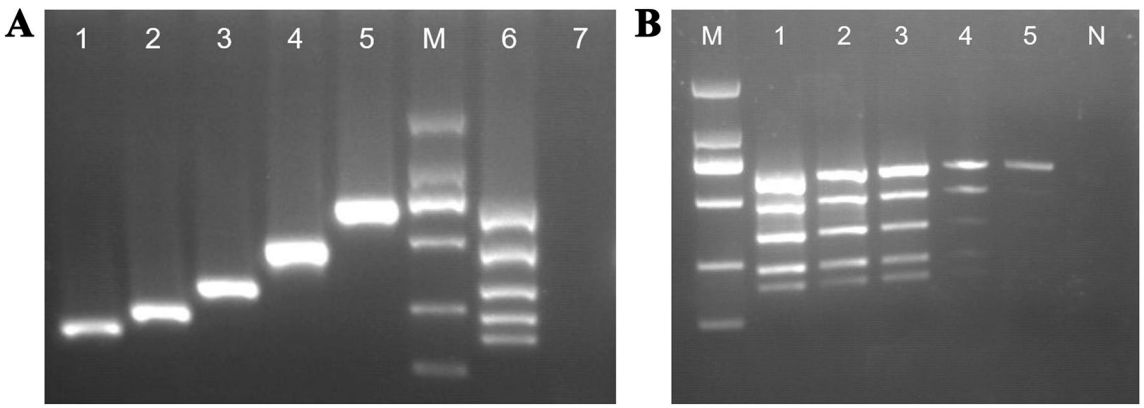

Fig. 2 Single and multiplex amplify assays. **a** Lane 1–5, single PCR amplification with species-specific primers for the detection of donkey, sheep, cattle, pig and horse respectively. Lane 6, multiplex PCR amplification with the mixture of five genome DNA and species-specific primer. Lane M, DNA Marker DL2000. Lane N, negative control. B: Sensitivity test of multiplex PCR assay for horse, pig, cattle, sheep and donkey. Lane M: DNA Marker DL2000. Lane 1, 20% concentration of the DNA from each species. Lane 2, 10% concentration of the DNA from each species. Lane 3, 1% concentration of the DNA from each species. Lane 4, 0.1% concentration of the DNA from each species. Lane 5, 0.01% concentration of the DNA from each species. Lane N: negative control

the sensitivities threshold of horse can even reach 0.01% (0.05 pg/μL) (Fig. 2b).

Real-time PCR

Specificity verification of reference primers

In order to develop a robust quantitative method, nuclear 18S rRNA gene was used to reference for a more accurate result. To investigate the specificity of the reference primers, the DNA from ten animal species, including *E. asinus, E. caballus, S. scrofa, O. aries, B. taurus, A. platyrhynchos domesticus, G. gallus domesticus, A. cygnoides orientalis* and *V. vulpes, C. coturnix*, was utilized respectively as template in the real-time PCR assay. Results showed that the reference primers could amplify the 18S rRNA gene and the Ct values were ranged from 17.39 to 25.42 (Fig. 3). No signal was found in negative control.

Construction of normalized standard curve

The standard curve normalized with 18S rRNA was proposed by the following equation:

$$\Delta Ct = Ct_{12S\ rRNA} - Ct_{18S\ rRNA}$$

The $Ct_{12S\ rRNA}$ and $Ct_{18S\ rRNA}$ represented the mean cycle thresholds for donkey specific primer and reference primer, respectively, obtained through the amplification of binary model mixtures (Fig. 4b and c). The normalized standard curve obtained from binary mixed DNA was plotted by mean ΔCt values and logarithm of 10-fold serially diluted donkey DNA percentages (100%, 10%, 1%, 0.1% and 0.01%). As shown in Fig. 4a, a high correlation coefficient ($R^2 = 0.9983$) and PCR efficiency (E = 103.52%) were observed.

The melt curve data showed that the amplicons with donkey-specific primer and with reference primer were both highly specific (Fig. 4d). The calculated formula of the standard curve was: $y = -3.2403 \times -3.7074$, therefore, the percentage of donkey meat adulterated by horse meat in this assay can be calculated with the follwing formula: $C\% = 10^{(\Delta Ct + 3.7074)/(-3.2403)} \times 100\%$.

Sensitivity test of the quantification method

To determine the sensitivity of quantification method, DNA of donkey was 10-fold serially diluted with horse DNA from 100 to 0.0001%. All of 7 gradients of diluted DNA were used as templates for the normalized real-time amplification. Result demonstrated that the detection limit of this method was 0.01% (w/w) (0.5 pg/μL).

Validation of the quantification method

To validate the quantification method we developed, the total DNA extracted from different ratios of six mixtures (mixed donkey meat into horse meat) of 90%, 50%, 25%, 10%, 5% and 1%. ΔCt values and the estimate content of donkey meat as well as the recovery rate were calculated as mentioned above. As showed in Table 4, bias of six adulterate samples was satisfied with acceptable criterion ($\leq \pm 25\%$) between actual and estimate values. Furthermore, Relative standard deviation (RSD) of Ct values was less than 1.81% and the recovery rate was ranged from 87.02 to 124.60%,

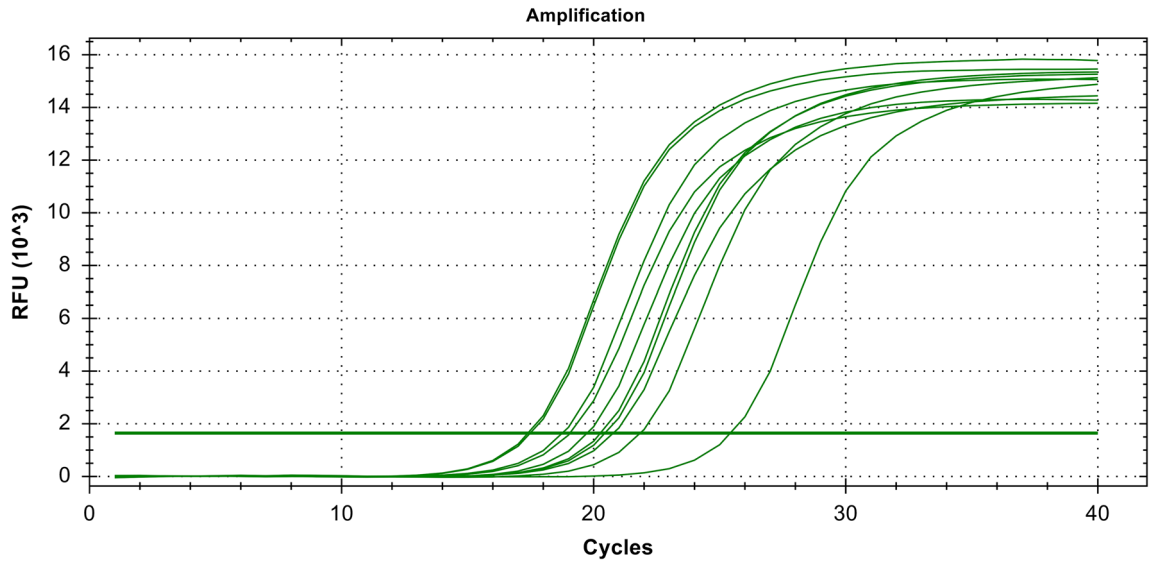

Fig. 3 Fluorescent amplification curve of reference primers for ten species including *E. asinus, E. caballus, S. scrofa, O. aries, B. taurus, A. platyrhynchos domesticus, G. gallus domesticus, A. cygnoides orientalis and V. vulpes, C. coturnix*

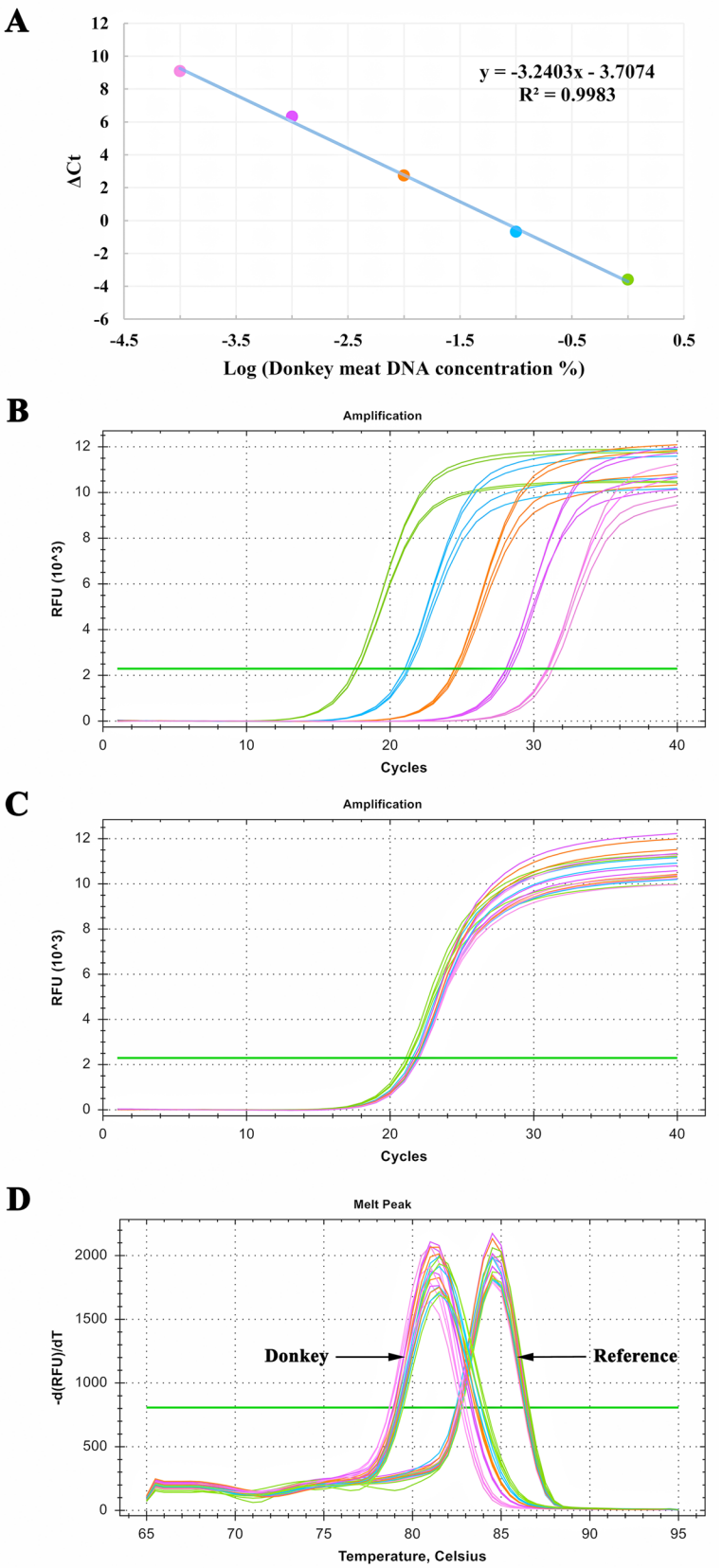

Fig. 4 Normalized standard curve for estimate the percentage of horse meat adulterated in donkey meat by using ΔCt method (**a**). Corresponding melt curve (**d**) and amplification curvesof binary meat mixtures (0.01 to 100% of donkey meat) by real-time PCR for donkey (**b**) and reference (**c**) systems

Table 4 The validations of the real-time PCR quantitative assay with simulate samples

Actual donkey meat (%, w/w)	Mean specific Ct value	Mean reference Ct value	Mean ΔCt value	Mean estimate donkey meat (%, w/w)	SD (%)	Recovery (%)	Error (%)
90.000	14.165	17.746	-3.581	91.382	8.522	101.535	1.54
50.000	14.833	17.587	-2.754	50.784	5.934	101.567	1.57
25.000	14.039	15.610	-1.571	21.908	3.913	87.632	−12.37
10.000	17.250	18.026	-0.777	12.460	1.770	124.602	24.60
5.000	16.404	15.700	0.704	4.351	0.444	87.022	−12.98
1.000	18.841	16.028	2.813	0.972	0.160	97.216	−2.78

demonstrating the improved method had high accuracy for estimating the content of donkey in horse meat in the range 1–90%.

Discussion

In recent years, frequent adulteration of meat, which was a kind of unfair market competition would lead to economical, religious and health problem to customers, had caused widespread concern in society [25]. It had been a very long history to raise donkeys in China. Nowadays, more than 9 million donkeys, almost 22% of the total donkey population around the world, are widely distributed in China [26]. Since donkey meat have good nutritional quality with high content of essential amino acids [27] and donkey hide is also the main raw material for the production of Colla Corii Asini which served as a traditional Chinese medicine. The demand for donkey continues to increase in recent years. However, in last four decades, the number of donkey decreased dramatically. These situations might be the main reasons for the presence of donkey-derived product adulteration. Donkey meat was adulterated with horse or fox meat as well as Asini Corii Collas was adulterated with skins or bones from other animals such as horses, cattle, and pigs [6, 28]. Accordingly, accurate qualitative or quantitative detection of adulterated meat species is important to the consumers for economic, health, and religious concerns. Several conventional and real-time PCR techniques had been developed, however, high possibility of false negative in conventional PCR technique limited its application. Moreover, the real-time PCR technique could not precisely estimate the content of target meat when extracted DNA seriously denatured or with some PCR inhibitors [23].

The conventional multiplex PCR technique developed in this study could be easy to achieve and did not required high-cost reagent or equipment, thus almost all of the laboratory could afford it. One person could easily do it in a half-day following three steps including extracting DNA, running PCR amplification program and agarose gel electrophoresis. Since cells had multiple copies of mitochondrial DNA, the mitochondrial 12S rRNA gene was selected as a suitable mocular marker. Our results indicated that all of the five pairs of species-specific primer were highly specific. Moreover, sensitivety test demonstrated that the sensitivity threshold of the multiplex PCR assay was 0.1% (5 pg/μL) for donkey, sheep, cattle and pig respectively, while the sensitivity threshold for horse was 0.01% (0.5 pg/μL), which were consistent with results in detection of pork adulteration [29]. However, the conventional multiplex PCR could not achieve quantitative detection of the content of donkey meat in foodstuff.

Accordingly, an improved real-time quantitative PCR technique was developed as a diagnostic tool to detect and quantify donkey meat. It is wellknown that the mitochondrion distributes unevenly in different tissues, moreover, different process or some plant source of ingredients added in may inhibit the PCR amplification. Therefore, the 18S rRNA was selected as eukaryotic control to normalize the standard curve which was helpful to obtain a more accurate and precies result [30]. This method showed a more acceptable result than other methods to construct standard curve [30]. The normalized standard curve developed in this assay indicated that the sensitivity threshold of this technique would be 0.01% (0.5 pg/μL), which was consistent with results about the analysis of pork in a beef product [30]. These results demonstrated that the normalized real-time quantitative PCRcould sensitively and quantitatively dectect the content of donkey meat in foodstuff. Simulated adulteration experiments revealed that this improved real-time quantitative PCR was capable to detect the content of donkey meat in commercial foodstuffs with high accuracy in the range 1–90% of adulteration.

Conclusion

In this study, a conventional multiplex PCR and normalized real-time PCR were firstly established to simultaneously detect five common type meats or quantitatively detect the content of donkey meat in foodstuff. The conventional multiplex PCR technique was proven to be specific and sensitive

to distinguish donkey from other four common adulterated species including sheep, cattle, pig and horse. An improved real-time PCR with the normalized standard curve could sensitively and quantitatively dectect the content of donkey meat in foodstuff. Moreover, simulate adulteration samples were successfully validated that the developed real-time PCR method could detect the quantification of donkey meat in the range of 1–90% with high recovery (87.02–124.60%). These results suggested that the conventional multiplex PCR and real-time quantitative PCR methods were alternative methods to qualitatively or quantitatively detect donkey-derived product adulteration in commercial foodstuff.

Acknowledgements We greatly appreciate the technical assistance by Ms. Pan Zhang for her help in DNA isolation and qRT-PCR assays.

Funding This work was funded by experimental technology research project of Zhejiang University (SJS201811).

Compliance with ethical standards

Conflict of interest All of the authors declare no conflicts of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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