

Evaluation of Methods of DNA Extraction from Down, and a Quantitative Real-time PCR Assay for Authenticity Control in Down Products

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ABSTRACT

Down, mainly duck down and goose down, is one of the most important products in the poultry industry. To improve the accuracy of identification of duck and goose down by quantitative real-time PCR (qPCR), and the efficiency of extracted DNA from down was evaluated and optimized using the template preparation methods, including four pretreatment methods (shredding [PA], shredding + magnetic bead homogenization [PB], shredding + manually grinding in liquid nitrogen [PC], and shredding + grinding by 6875 Freezer [PD]) and three extraction methods (a magnetic bead adsorption method [MA], a membrane adsorption-elution method [MB], and a Chelex® 100 Resin method [MC]), and their combinations. The results showed that high-intensity grinding, and grinding in liquid nitrogen can help the improvement of DNA yield, therefore, PB, PC, and PD were superior to PA both in DNA concentration and purity. However, in the subsequent qPCR analysis, the DNA of the highest quantity and purity from PD indicated the lowest positive detection rate. According to the results of the current study, the extraction method had a greater impact on the quality of DNA than the pretreatment method. Although the concentrations and purities of the templates obtained by MA, MB, and MC were varied greatly, all the templates could meet the needs in the following qPCR assay. Furthermore, considering the simplicity of the methods, PA + MC was chosen as the most convenient and efficient combination to extract DNA from down. A quantitative calculation method for the identification of the authenticity of down products was established. Although quantification results could not estimate the target content accurately, they reflected the trend in the content. Nevertheless, the method may be useful as an alternative tool for qualitative and quantitative quality control of the down products.

Keywords: Authenticity discrimination, Down, DNA extraction, Quantitative detection, Quantitative PCR

INTRODUCTION

Duck down and goose down are two main kinds of down products on the market. There is no significant difference in their appearance, but as a natural insulator, goose down is better than duck down, so, the price is also higher than that of ducks. Consequently, the adulteration of duck down or landfowl down in goose down has occurred. In addition to duck and goose down, down also exists in many other forms, such as whole waterfowl feather, broken and damaged waterfowl feather, whole landfowl feather, broken and damaged landfowl feather, plumules, down fibers, feather fibers, quill feathers, and residual matter. The most commonly used method for classification of all types of downs is based on morphological differences, which could be identified using the naked eye

or microscopic observation. There are several standards for the classification and determination of the quantitative composition of feather and down, such as the European standard BS-EN-12131: 2018 (2018), the Chinese standards FZ/T 80001-2002 (2002), and GB/T 10288-2016 (2016) that also provide the basis for species identification of downs and feathers. However, since the description in the standards standard is more of a written description, it is difficult for the operator to master morphological identification.

Chen et al. (2013) designed the duck and goose species-specific primer/probe sets to establish the quantitative real-time PCR (qPCR) method for the identification of duck down and goose down. The qPCR method overcomes the subjectivity of the manual method

and could be chosen as an auxiliary for detection. In this regard, DNA of sufficient yield and quality is the crucial starting material for the qPCR analyses. As Chen et al. (2013) mentioned, although the quality of the DNA could meet the needs of qPCR testing, the method of DNA extraction with sodium dodecyl sulfate (SDS)-guanidine thiocyanate and β -mercaptoethanol was time-consuming and laborious; and of all the steps, a water bath alone took about 6 hours, and DNA precipitating from alcohol took even a night. Although reports on DNA isolation from down products are rare, there are some studies on DNA extraction from bird feathers (Speller et al., 2011; Boonseub et al., 2012; Presti et al., 2013), animal hair (Subramanian et al., 2005; Alberts et al., 2010; Henry and Russello, 2011), and human hair (Haines and Linacre, 2016; Grisedale et al., 2018). Among the methods, various commercially available DNA extraction kits were a common choice (Tomasek et al., 2008; Boonseub et al., 2012; Presti et al., 2013). In addition, Tris-HCl based lysis method (Bello et al., 2001), simple alkaline extraction method (Malagó et al., 2002), and the Chelex®100 Resin method (Tomasek et al., 2008) were often used as the alternatives. However, in previous studies, the pretreatment processing of the sample before DNA isolation was rare. The current study aimed to adapt and compare a variety of pretreatment methods to investigate the accuracy of qPCR method to identify duck and goose down in addition to the comparison of DNA extraction methods. Finally, an optimum combination of pretreatment procedure and DNA isolation method were presented. Furthermore, taking the advantage of the quantifiable ability of the qPCR method, this study presented a way to calculate the proportion of goose down and duck down or any other landfowl down in the down products, which would be beneficial to quality control.

MATERIAL AND METHODS

Sample selection and preparation

The down and feather samples were provided by the Zhejiang Zhongda Technical Import and Export Group Co., Ltd, and the Microbiological Laboratory of Technical Center of Hangzhou Customs. Three samples of Beijing white duck down, Polish white duck down, and Northeast white goose down were chosen for a comparative test on the various pretreatment and DNA extraction methods. To prepare binary species down mixtures (duck down in goose down), Polish white duck down and China Northeast white goose down were used as materials. Six different percentages, 0, 10, 20, 30, 40, 50% (w/w) of

duck down were prepared for each duck-geese binary mixture, to a final weight of 1 g.

Pretreatment methods for DNA extraction

Before DNA extraction, the down was cut as small as possible by scissors, and then the following pretreatments were performed.

Preprocessing A (PA)

No further pretreatment was followed, and DNA was extracted directly according to the kit.

Preprocessing B (PB)

The shredded down with magnetic beads (ϕ 3 mm; Sigma, USA) was homogenized on a high-throughput bead beating homogenizer (Bertin Precellys Evolution, Bertin Technologies, France) using 5500 r/min for 20 seconds, and was repeated 4-6 times.

Preprocessing C (PC)

The shredded down was manually ground in liquid nitrogen until it was fractured.

Preprocessing D (PD)

The shredded down was ground by 6875 Freezer/Mill® High Capacity Cryogenic Grinder (SPEX Sample Prep, USA). The procedure started with pre-cooling the instrument and sample for 7 minutes, followed by grounding the sample for 2 minutes at 12 CPS (cycle per second) and cooling the instrument and sample again for 2 minutes. After the pretreatment, 50 mg material was taken from each sample for DNA isolation by Wizard Magnetic DNA Purification System for Food (FF3750, Promega, USA) according to the manufacturer's instructions. Finally, the DNA was dissolved in 50 μ L H₂O.

DNA extraction methods

An ideal method was selected as a unified preprocessing method from the four pretreatments of A, B, C, and D by comparing DNA yield, purity, and the results of qPCR. Based on it, in the following test, the effects of three different DNA extraction methods were compared.

Method A

Method A (MA) involved Wizard Magnetic DNA Purification System for Food (FF3750, Promega Corporation, USA) extraction kit. This DNA purification kit uses the magnetic beads adsorption method for DNA isolation. For each extraction test, 50 mg of down sample was used. Finally, DNA was dissolved in 50 μ L water. The whole process took 1.5 to 2 hours.

Method B

In Method B (MB), QIAamp DNA Mini Kit (50) (51304, QIAGEN GmbH, Germany) was used. It's a silica

membrane column-based DNA purification system. For each test, 25 mg of sample was used. DNA was dissolved in 25 µL water. Including 3 hours of incubation for complete lysis. The process took about 4.5 hours.

Method C

Chelex® 100 Resin (Sigma, USA) method was used in method C (MC). A 50 mg sample was added to the microcentrifuge tube and 500 µL 5% w/v Chelex® 100 Resin and 10 µL 10 mg/mL proteinase K (TaKaRa, Dalian, China) pipetted into the tube. Then, the tube was incubated for 30 minutes at 56°C followed by a 15 minutes incubation at 99°C. After cooling, the sample was vortexed briefly and centrifuged at 20000 × g for 10 minutes. The supernatant was used as the DNA sample in the following test. The process took about 1 hour.

DNA quality assessment

DNA concentrations were measured spectrophotometrically by A₂₆₀ nm of NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). The purity was assessed by measuring the ratio of absorbance at 260 nm and 280 nm, respectively. A_{260/280} is a relative absorbance reading, indicating DNA purity, and should ideally be 1.8 for most molecular biology applications. In addition, the DNA quality was also assessed by the downstream fluorescence PCR. All DNA isolation and qPCR experiments were repeated at least three times.

Real-time PCR amplification

The duck, goose, and chicken species-specific qPCRs were carried out according to a Chinese trade standard SB/T 10923-2012 (2013). The primers and fluorogenic probes used in the qPCR were based on the standard with some modifications (Table 1) and purchased from TaKaRa (Dalian, China). Real-time PCR was performed with an initial denaturation of 95°C for 10 seconds, followed by 40 cycles at 95°C for 5 seconds and 60°C for 23 seconds

(LightCycler 480, Roche Applied Science, Penzberg, Germany). The qPCR mixtures contained 10 µL Premix Ex Taq (TaKaRa, Dalian, China), 0.4 µL of each primer (10 pmol/µL), 0.4 µL of probe (10 pmol/µL), 2 µL of each template DNA, and water in a total volume of 20 µL (Wu et al., 2017). All the qPCR experiments were repeated at least three times.

Construction of the standard curve and data analysis

To assess the efficiency, linear range, and analytical sensitivity of the qPCR, the diluted purified DNA from duck and goose was used to construct separate standard curves (Pegels et al., 2014). DNA was isolated from the meat of domestic duck and Chinese domestic goose individually by the combination of PB and MA. The initial concentration of the two kinds of DNAs was standardized to 20 ng/µL, from which a serial dilution was carried out (dilution factor 1:4). After diluting by water, seven concentrations (20, 5, 1.3, 0.3, 0.08, 0.02, 0.005 and 0.0001 ng/µL) were prepared for duck and goose DNAs. Primer efficiencies were calculated using standard curves. The efficiencies for duck and goose were 1.588 and 1.612, respectively.

The amount of target DNA in an unknown sample was then measured by extrapolation of the threshold cycle (Ct) value obtained in the unknown sample in the corresponding standard curve of Ct values generated from known DNA percentages of the target species. The correlation between the variables, Ct, and concentration ([I]) is semilogarithmic:

$$Ct = b * \log [DNA \text{ concentration}] + a$$

Where, b is the slope and a denotes the intercept (Pegels et al., 2014).

The linearity of the model was verified by regression analysis (ANOVA). In the coefficient of correlation, r, expresses statistically the correlation between test results obtained (Ct values) and DNA concentration in the serial dilution, whereas the coefficient of determination, r², indicates the correlation quantitatively (Pegels et al., 2014).

Table 1. Primers and probes for detection of duck, goose, and chicken by qPCR.

Target species	Nucleotide sequence (5'-3') and fluorescent label	Amplicon size (bp)	Reference
Domestic duck (<i>Anas platyrhynchos</i> and <i>Anas poecilorhyncha</i>)	F: GGCCACACAAATCCTCACAG R: TGTGTTGGCTACTGAGGAGAAA P: FAM-CCTACTGGCTATGCACTACACCGCAGAC-Eclipse	85	
Chinese domestic goose and European domestic goose (<i>Anser cygnoides</i> and <i>Anser anser</i>)	F: R ^b GACAATCCAACCTTAACCCGA R: GGACTAGGGTGATTCCTGCA P: FAM-CCATCCACTTCCT ^c CTGCCCTTCCTA-Eclipse	77	SB/T 10923-2012 (2013)
Domestic chicken (<i>Gallus gallus domesticus</i>)	F: CGACAACCCAACCTTACC R: AGGAAGGTGAGGTGGATGATA P: FAM-ACACTTCTCCTCCCCTTTGCAATCGC-Eclipse	89	

F: Forward primer, R: Reverse primer, P: Probe, bp: Base pair, ^b^cModifying the original base A into R

RESULTS

Comparison of different preprocessing

The DNA extracted with PA (treated by shear only) was very low in concentration, about 2.0 ng/ μ L, and also poor in purity, with the value of $A_{260/280}$ between 2.4 and 3 (Table 2). After pretreated with magnetic bead grinding (PB) or liquid nitrogen grinding (PC), the DNA concentration increased to about 4 ng/ μ L, while the values of $A_{260/280}$ were between 1.5 and 2.3, better than PA. Synchronizing liquid nitrogen and physical grinding treatments, the PD process greatly increased the DNA concentration to more than 20 ng/ μ L, and the DNA was of higher purity ($A_{260/280} = 1.6$ to 2.1) than other treatments (Table 2).

For PA, PB, and PC, the results of qPCR were consistent with the purity and quantity of the DNA (Table 2). As long as the concentration and purity of the DNA were low, the corresponding Ct value was high; otherwise, it was relatively low. For the same sample, the corresponding Ct value gradually decreased for pretreated with PA, PB, and PC, especially in duck-species detection for the sample of Beijing white duck and Polish white duck, where the Ct of PA was about 37, the Ct of PB and PC ranged 31.1-32.5. When in goose-species detection for Northeast white goose, the Ct of PA was up to 37.7, but if it was pretreated by PB or PC, the Ct dropped to around 30; which fully demonstrated the importance of the purity and quantity of DNA for PCR detection. However, for the PD method, it was different. Though the DNA concentration was significantly higher than that of others, and the DNA purity was also pretty good (Table 2), the qPCR results were out of expectation, where no positive result was obtained in detecting duck species, but only the Ct value of goose detection was better than that of other methods.

According to the quality of the DNA, the results of qPCR, and the convenience of operation, PB was chosen as a unified preprocessing way in the following experiments for comparison of different DNA extraction methods (MA, MB, and MC), unless noted otherwise.

In addition, based on the results of species-specific identification by qPCR (Table 2), it was found that no positive results were obtained when detecting chicken DNA in either duck down or goose down, and for the samples of duck down, the positive signals were only observed in the duck-specific test. However, for goose down samples, both goose and duck-derived DNA were detected, but the content of the duck was lower with the Ct value close to 35.

Comparison of different DNA isolation methods

With the same pretreatment, the efficiency of different DNA extraction methods was compared and the DNA concentration and purity obtained with each method were presented in Table 3. MA yielded the lowest amounts of DNA, with concentrations between 3.1 ng/ μ L to 4.6 ng/ μ L. MB increased the concentration up to 13.4 ng/ μ L, and the DNA was of higher purity, with the $A_{260/280}$ values closer to 1.8. DNA obtained by MC was of the largest amount, with the concentrations ranging from 153.4 ng/ μ L to 188.2 ng/ μ L, but due to the lack of purification processing steps, the purity was quite lower ($A_{260/280}=1.1$ to 1.2).

In the downstream fluorescence qPCR test, it was confirmed that for the quality of DNA, purity was as important as concentration. Although the concentration of DNA obtained by MC was high, the Ct values were not superior to the corresponding ones of MA and MB. For the same sample, the Ct values of MB in Table 3 were always the lowest, and the values of MC were always the highest; and for all the three samples, the values of MA in Table 3 were close to the corresponding ones in Table 2, around 30, which indicates that the performance of the method was stable. The species detection results presented in Table 3 were in accordance with the ones in Table 2. In the duck down, only duck DNA was detected, however, in the goose down, not only goose DNA but also duck DNA were detected. Furthermore, the Ct value of the duck detection in the goose down lowered to 30.1.

Based on the above-mentioned results, it was suggested that the extraction methods have more influence on the DNA isolation efficiency than the preprocessing methods. Therefore, to simplify the operation, two new combinations, PA + MB and PA + MC, were tested, in which before DNA extraction, the down was only cut to small pieces by scissors. The results of the DNA quality are shown in Table 4. Considering DNA concentration and the value of $A_{260/280}$, the combinations of PA + MB and PA + MC (Table 4) were not as effective as the combinations of PB + MB and PB + MC (Table 3) in extraction DNA from the down. However, the results of the detection of species (Table 4) showed that the quality of DNA isolated by PA + MB and PA + MC can meet the requirements of qPCR detection. Unexpectedly, the Ct values of the combination of PA + MC (Table 4) were lower than the corresponding values of PB + MC (Table 3) and PA + MB (Table 4) but close to that of PB + MB (Table 3). According to these results, the combination of PA + MC was considered a simple and effective way to obtain DNA

from the down; therefore, in the following experiments, DNA of down was extracted by this combination.

Table 2. Effects of different pretreatment methods on the quality of DNA extracted from down samples

Sample	Pretreatment	DNA concentration (ng/μL)	A _{260/280}	qPCR (Ct mean ± SD) ^a		
				Chicken	Duck	Goose
Beijing white duck down	PA	2.1 ± 0.8	3.0 ± 0.6	— ^b	36.7 ± 0.9	—
	PB	3.8 ± 0.4	1.6 ± 0.5	—	31.1±2.5	—
	PC	3.7 ± 1.5	2.1 ± 0.3	—	32.5±1.6	—
	PD	27.9 ± 2.2	2.1 ± 0.4	—	—	—
Polish white duck down	PA	1.6 ± 0.4	2.4 ± 0.5	—	37.0 ± 0.8	—
	PB	5.7 ± 0.9	1.7 ± 0.1	—	30.5 ± 1.3	—
	PC	3.4 ± 1.0	2.1 ± 0.4	—	32.1 ± 0.8	—
	PD	20.8 ± 1.4	2.0 ± 0.1	—	—	—
Northeast white goose down	PA	1.9 ± 0.2	2.5 ± 0.5	—	—	37.7 ± 1.3
	PB	4.3 ± 1.4	2.3 ± 0.3	—	33.9 ± 0.6	30.4 ± 1.8
	PC	4.7 ± 0.9	1.5 ± 0.1	—	34.9 ± 0.7	30.8 ± 1.5
	PD	48.7 ± 1.3	1.6 ± 0.2	—	—	27.7 ± 0.2

qPCR: Quantitative real-time PCR, Ct: Threshold cycle, SD: Standard deviation. PA: Shredding, PB: Shredding + magnetic bead homogenization, PC: Shredding + manually grinding in liquid nitrogen, PD: Shredding + grinding by 6875 Freezer. In the experiments, DNA was all extracted by the MA method (a magnetic bead adsorption method). ^a Average Ct value ± SD shown from triplicate PCR reactions from each DNA extraction. ^b Not detected (no positive signal after 40 PCR cycles).

Table 3. Effects of different DNA extraction methods on the quality of DNA extracted from down samples

Sample	Extraction method	DNA concentration (ng/μL)	A _{260/280}	qPCR (Ct mean ± SD) ^a		
				Chicken	Duck	Goose
Beijing white duck down	MA	3.1 ± 1.1	2.0±0.6	— ^b	31 ± 1.6	—
	MB	11.4 ± 3.1	1.7±0.3	—	26.7 ± 1.5	—
	MC	153.4 ± 23.2	1.1±0.3	—	32.4 ± 2.5	—
Polish white duck down	MA	4.8 ± 1.4	1.9±0.5	—	29.2 ± 0.8	—
	MB	13.4 ± 4.7	1.8±0.4	—	26.4 ± 1.1	—
	MC	167.4 ± 31.8	1.2±0.2	—	31.6 ± 3.5	—
Northeast white goose down	MA	4.6 ± 0.8	1.5±0.6	—	32.9 ± 1.4	30.3 ± 0.2
	MB	10.7 ± 1.9	1.7±0.4	—	30.1 ± 0.8	25.3 ± 1.3
	MC	188.2 ± 46.8	1.1±0.2	—	34.1 ± 1.8	31.3 ± 2.7

qPCR: Quantitative real-time PCR, Ct: Threshold cycle, SD: Standard deviation. All the samples were pretreated by PB (shredding + magnetic bead homogenization). MA: a magnetic bead adsorption method; MB: a membrane adsorption-elution method; MC: a Chelex® 100 Resin method. ^aAverage Ct value ± SD shown from triplicate PCR reactions from each DNA extraction. ^b Not detected (no positive signal after 40 PCR cycles).

Table 4. Effects of combinations of PA + MB and PA + MC on the quality of DNA extracted from down samples

Sample	Methods	DNA concentration (ng/μL)	A _{260/280}	qPCR (Ct mean ± SD) ^a		
				Chicken	Duck	Goose
Beijing white duck down	PA+MB	6.4 ± 2.1	2.1 ± 0.4	— ^b	26.5 ± 0.8	—
	PA+MC	79.1 ± 18.1	1.1 ± 0.2	—	25.4 ± 0.5	—
Polish white duck down	PA+MB	9.1 ± 1.5	2.4 ± 0.6	—	27.4± 2.4	—
	PA+MC	89.4 ± 16.5	1.2 ± 0.1	—	25.1 ± 1.1	—
Northeast white goose down	PA+MB	5.7 ± 1.9	2.3 ± 0.9	—	33.2 ± 0.8	28.1 ± 1.1
	PA+MC	75.7 ± 1.4	1.0 ± 0.9	—	30.3 ± 1.7	25.2 ± 0.9

qPCR: Quantitative real-time PCR, Ct: Threshold cycle, SD: Standard deviation. All the samples were pretreated by PA. PA: Shredding, MB: A membrane adsorption-elution method, MC: A Chelex® 100 Resin method. ^aAverage Ct value ± SD shown from triplicate PCR reactions from each DNA extraction, ^b Not detected (no positive signal after 40 PCR cycles)

Species detection for eleven samples

By the combination of PA + MC, DNA of 11 down samples were extracted. The concentrations of the DNA were high, all more than 70 ng/ μ L, but the purities were low, none of the values of $A_{260/280}$ higher than 1.5. However, the Ct values of the target species detection for the 11 samples ranged from 25.7 to 33.7, indicating that the quality of the DNA can meet the needs of qPCR detection. The species detection of the 11 samples showed that no chicken DNA was detected in all the samples; in 8 ducks down samples, only duck DNA was detected; and in 1 duck down and 2 goose down samples, both duck and goose DNA were detected. Detection of duck down from goose down is usually suspected of adulteration, if the amount of duck down greatly exceeds the labeled amount. Conversely, detection of goose down from duck down might be due to unintended cross-contamination.

Duck and goose standard curve and determination of mixing ratios

Eight 4-fold dilution series of duck and goose DNA starting from 20 ng/ μ L were prepared for the construction of standard curves in qPCR. Considering 2 μ L template DNA used in each qPCR mixture, the detection limits for duck and goose specific PCR were both 0.01 ng (Table 1, Figure 1). To test the linearity, Ct values were plotted versus the logarithm of the DNA concentrations (Figure 1). The coefficient values (r^2 values) of duck- and goose-specific standard curves were 0.9834 and 0.9806, respectively, which indicated 98.34% and 98.06% of the variations in Ct values, individually in Figure 1a and figure 1B, can be attributed to the variations in the logarithm of DNA concentrations.

According to a certain proportion (Table 6), the mixed samples of Polish white duck down and Northeast white goose down were prepared. The DNA extracted from the binary mixtures was detected by duck and goose-specific qPCRs, respectively. Based on the Ct values of the fluorescent PCR tests and the standard curves in figure 1, DNA concentrations of duck and goose in the mixtures were calculated. Furthermore, 3.7%, 10.9%, 20.6%, 31.6%, 43.9%, and 56.6% proportions of duck component were calculated in the six samples with an initial theoretical proportion of 0, 10%, 20%, 30%, 40% and 50%, respectively. The Northeast white goose down used in this study was 90% down meaning that at least 90% of the ingredients in the down were goose down clusters and goose down fibers, and the remaining 10% might be the goose feather, the other waterfowl or landfowl feather, or feather fibers. Assuming that 3.7% duck component in 100% goose down was true, the theoretical values of the 10-50% duck mass percentage needed to be adjusted. The original contents 10% and 20% should be adjusted to 13.3% ($[10+90*0.037]*100\% = 13.3\%$) and 22.9% ($[20+80*0.037]*100\% = 22.9\%$), respectively, and the other values in the same column could be calculated in the same way. When the proportion of duck down was between 30% and 40%, the calculated values were relatively close to the corresponding adjusted theoretical values, with the gaps of 3.1% and 4.0%. But when the duck down content was less than 30% or greater than 40%, the difference between the two corresponding values increased. Based on the calculation method, it was found that the proportions of duck down in the white goose down and Grey goose down in Table 5 were as high as 9.5% and 6.3%, respectively.

Table 5. Detection of goose, duck, and chicken species in 11 down samples by qPCR

Sample	DNA concentration (ng/ μ L)	$A_{260/280}$	qPCR (Ct mean \pm SD) ^a		
			Chicken	Duck	Goose
Anhui white duck down 277	96.2 \pm 24.6	1.2 \pm 0.2	— ^b	27.7 \pm 2.2	—
Anhui white duck down 352	109.8 \pm 33.8	1.3 \pm 0.3	—	25.7 \pm 2.1	—
Guangxi white duck down	112.8 \pm 21.9	1.1 \pm 0.1	—	30.6 \pm 2.2	—
Guangxi Guigang white duck down	86.9 \pm 13.3	1.4 \pm 0.3	—	28.4 \pm 2.8	33.2 \pm 2.8
Grey duck down 362	117 \pm 23.7	1.2 \pm 0.1	—	30.4 \pm 2.6	—
Lu'an grey duck down 309	94.4 \pm 22.6	1.4 \pm 0.2	—	29.8 \pm 1.3	—
Guangxi Guigang grey duck down	106.1 \pm 33.4	1.2 \pm 0.4	—	29.2 \pm 2.7	—
Chaozhou grey duck down	88.7 \pm 20.5	1.2 \pm 0.1	—	30.9 \pm 2.1	—
Guangdong grey duck down	118.4 \pm 34.1	1.3 \pm 0.2	—	28.6 \pm 1.7	—
White goose down	73.2 \pm 23.0	1.4 \pm 0.3	—	31.1 \pm 2.1	27.3 \pm 1.6
Grey goose down	100.4 \pm 19.3	1.4 \pm 0.4	—	33.7 \pm 1.3	28.9 \pm 2.4

qPCR: Quantitative real-time PCR, Ct: Threshold cycle, SD: Standard deviation. DNA was extracted by the combination of PA (shredding) + MC (a Chelex® 100 Resin method). ^aAverage Ct value \pm SD shown from triplicate PCR reactions from each DNA extraction; ^bNot detected (no positive signal after 40 PCR cycles).

Table 6. Calculation for duck down percentage in six mixed duck-geese down samples

No.	Sample	Duck		Goose		Percentage of duck down by calculation ^b	Adjusted percentage of duck down in theory	[(b-c)/c]×100 ^c
	Percentage of duck down in theory	Ct value ^a	DNA concentration (ng/μL)	Ct value ^a	DNA concentration (ng/μL)			
1	0%	29.8	0.081	23.9	2.12	3.7%	3.7%	--
2	10%	28.1	0.18	24.7	1.45	10.9%	13.3%	-18.0%
3	20%	27.1	0.28	25.3	1.08	20.6%	22.9%	-10.0%
4	30%	26.9	0.31	26.3	0.67	31.6%	32.6%	-3.1%
5	40%	26.6	0.36	27.1	0.46	43.9%	42.2%	4.0%
6	50%	26.0	0.47	27.6	0.36	56.6%	51.9%	9.1%

Ct: Threshold cycle, ^a Mean value of three results, ^b According to the concentrations of duck and goose, ^c Difference between the calculated value and the adjusted value in theory

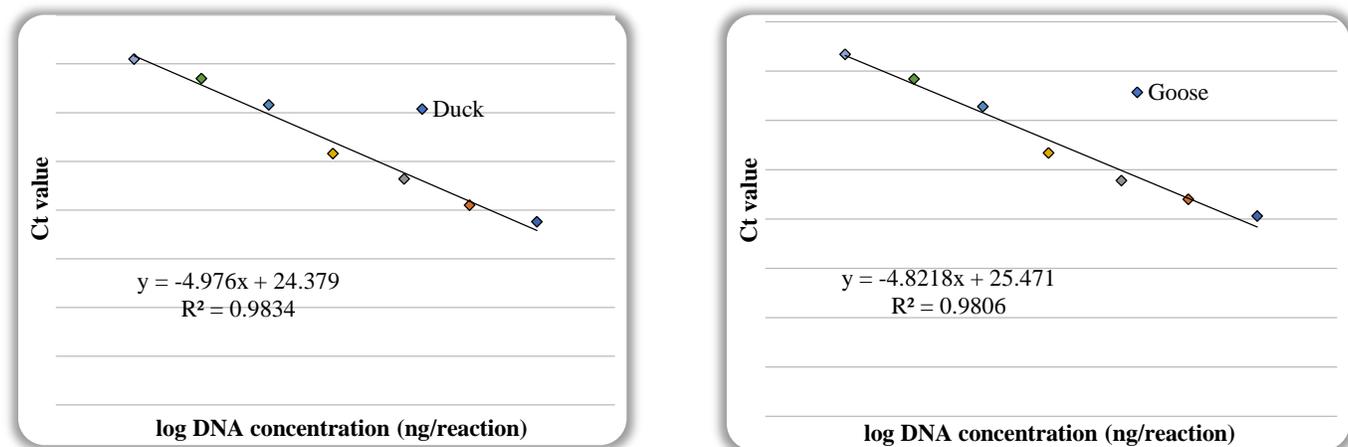


Figure 1. Amplification curve of DNA concentrations against the cycle threshold (Ct) values. A: For detection of duck; B: For detection of goose

DISCUSSION

DNA of sufficient yield and quality is the crucial starting material for molecular analyses. For this goal, four pretreatments and three DNA extraction methods were assessed. In the pretreatment process, except for PD, other treatments could do little to improve the concentration and purity of DNA. However, in addition to the quantity and purity of the template, the integrity of the DNA, and the presence or absence of inhibitors, all of these can affect subsequent PCR amplification. Therefore, although yielding the largest amounts of DNA and the DNA of higher purity, PD treatment, on the contrary, led to a decrease in the detection rate in avian-specific qPCR assay. Then, for confirmation, PD was repeated three more times, but the corresponding qPCR detection rate was still low, with 3 positive results out of 12 tests (data have not been shown). The PD treatment made all down samples into a fine powder, which facilitated the release of DNA from the cell, meanwhile broke the long strand DNA into small fragments. As a result, despite the high

concentration and purity of the template and the short length of the PCR amplicons (Table 1), the primer pairs still failed to amplify the target fragments. Physical grinding in pretreatment, even with the supporting equipment, was labor-intensive and time-consuming, and compared with the extraction process, the pretreatment process had little effect on improving DNA quality. Therefore, except for the shredding step, other pretreatment operations were omitted.

Chen et al. (2013) extracted DNA by SDS-agmatine thiocyanate-β-mercaptoethanol method at a concentration between 20 ng to 200 ng, which is similar to the DNA concentration obtained in this study. DNA in animal fiber mainly exists in the hair follicle part, and there is a small amount of mitochondrial DNA in the hair shaft. Since then, Chen et al. (2013) only chose the down with hair follicle for the experiment, and only the hair follicle part in the sample was used to extract DNA. In the present research, the whole down was used for DNA extraction, and regardless of whether it had a hair follicle or not. However, the randomness in sampling simplifies the

operation and is more in line with the needs of practical detection.

In addition to easy and rapid performance and inexpensive, an optimal DNA preparation method for routine testing should also avoid the use of hazardous chemicals (Tomasek *et al.*, 2008). In this study, two kit methods and Chelex® 100 Resin were chosen and evaluated. Compared with the kits, Chelex® 100 Resin treatment was easy for operation and yielded the largest amounts, but lowest purity of DNA (Table 3 and 4); nevertheless, the template DNA could satisfy the following qPCR detection. Similarly, Tomasek *et al.* (2008) also considered Chelex® 100 Resin the best option for extracting virus DNA from avian feathers when compared with NucleoSpin® Tissue Kit and DNazol® Direct kit methods. Here, according to the results, the combination of PA + MC was the best way to extract DNA from down.

The common goose down mainly comes from Chinese domestic goose and European domestic goose. Therefore, to enable to cover the two geese, the primer and probe sequences in the original standard were modified (Table 1). The copy numbers of the mitochondrion in muscle and down may be different, but due to the lack of 100% goose down, two standard curves were made by the DNA extracted from the meat of duck and goose. However, the duck down percentages of the binary mixtures calculated based on the standard curves were approximately close to the pre-set values (Table 6). In real-time PCR assay, in addition to DNA concentration, Ct value may also reflect the quality of DNA purity. DNA extracted from down by the combination of PA + MC might be lower in purity and higher in Ct value, which may lead to a deviation when measuring DNA concentration using the standard curve established with the DNA extracted from meat by the method of PB + MA. But instead of the absolute DNA concentration, the final value is a ratio of concentration, so the deviation can be eliminated.

CONCLUSION

This preliminary study demonstrated the potential application of qPCR for the identification of goose, duck, or chick down. This method can be used as an auxiliary method, especially, when morphological detection is difficult, such as using the young duck's down as goose down.

DECLARATIONS

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Authors' contributions

Shan Wu and Xiaofeng Zhang designed the research; Huizhen Yu, Yongqiang He, Mingzhe Zhang, Wenxiu Yin, Quan Zhang, Chao Sun, Zhe Chen, and Xufang Shen performed the research and analyzed the data; Shan Wu wrote the manuscript; Xiaofeng Zhang and Huizhen Yu have taken part in the revision of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethical considerations

Authors have checked ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy).

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