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ORIGINAL ARTICLE

A nuclear DNA-based species determination and DNA quantification assay for common poultry species

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Abstract DNA testing for food authentication and quality control requires sensitive species-specific quantification of nuclear DNA from complex and unknown biological sources. We have developed a multiplex assay based on TaqMan® real-time quantitative PCR (qPCR) for species-specific detection and quantification of chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), and turkey (*Meleagris gallopavo*) nuclear DNA. The multiplex assay is able to accurately detect very low quantities of species-specific DNA from single or multi-species sample mixtures; its minimum effective quantification range is 5 to 50 pg of starting DNA material. In addition to its use in food fraudulence cases, we have validated the assay using simulated forensic sample conditions to demonstrate its utility in forensic investigations. Despite treatment with potent inhibitors such as hematin and humic acid, and degradation of template DNA by DNase, the assay was still able to robustly detect and quantify DNA from each of the three poultry species in mixed samples. The efficient species determination and accurate DNA quantification will help reduce fraudulent food labeling and facilitate downstream DNA analysis for genetic identification and traceability.

Keywords Multi-species identification · Multi-species DNA quantification · Food safety · Food testing · Animal forensic science

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Introduction

Multiple approaches have been used for meat authentication (Primrose et al. 2010) with DNA analysis being the most common for species determination (Woolfe and Primrose 2004; Fajardo et al. 2010; Ballin 2010; Lockley and Bardsley 2000). Compared to other meat species, there are fewer poultry-specific assays for food authentication and quality control (Stamoulis et al. 2010). Most poultry DNA-based assays utilize avian mitochondrial genes and do not have a nuclear DNA quantification component (Dalmasso et al. 2004; Girish et al. 2007; Stamoulis et al. 2010; Kocher et al. 1989; Herman 2004; Haunshi et al. 2009; Girish et al. 2011). The integration of species determination and nuclear DNA quantification into a single assay will streamline downstream genotyping analysis, and improve the quality of the DNA profiles while conserving reagents (Evans et al. 2007; Lindquist et al. 2011; Kanthaswamy et al. 2012). Many of the DNA-based food authentication assays are multiplexed end-point PCR assays (Dalmasso et al. 2004; Zha et al. 2010; Ghovvati et al. 2009; Rodriguez et al. 2003). A majority of these protocols require subsequent steps such as restriction enzyme digestion or gel electrophoresis for species identification after PCR. End-point PCR assays with a quantification component have been developed, but amplicon quantities are typically determined using imaging software analysis of fluorescence intensities of electrophoresis gel bands (Soares et al. 2010).

Like end-point PCR, real-time quantitative PCR (qPCR) technology allows multiplexing, and is amenable to detecting multiple DNA targets that allow the simultaneous identification of DNA originating from multiple species. Moreover, the real-time DNA quantification feature of qPCR technology facilitates the efficient and accurate quantification of template DNA. While simplifying data collection and analysis, qPCR's ability to amplify, identify, and quantify in a single step effectively minimizes turnaround time and exposure to contamination and errors (Kanthaswamy et al. 2012).

Of the few poultry assays developed, most rely on mitochondrial DNA (mtDNA) genes as markers for detection (Girish et al. 2007; Haunshi et al. 2009; Herman 2001; Soares et al. 2010). Since mtDNA occurs in high copy numbers in each cell and is able to withstand degradation and environmental challenges, it is well suited for use in food authentication assays. MtDNA assays have already been developed and used for species testing in food safety management and fraudulence using both end-point PCR and qPCR techniques (Dooley et al. 2004; Köppel et al. 2009; Woolfe and Primrose 2004; Dalmasso et al. 2004; Zha et al. 2010; Rodriguez et al. 2003; Fumiere et al. 2006; Eugster et al. 2009; Tanabe et al. 2007). Despite mtDNA's durability (Hajibabaei et al. 2007), nuclear loci are more advantageous for DNA quantification because the diploid (instead of multiple) copy number (Kanthaswamy et al. 2012; Walker et al. 2004) makes it more predictive of nuclear DNA profiling success and more relevant to identity testing and sample traceability than multi-copy and/or non-nuclear markers (Alonso et al. 2004; Timken et al. 2005). Targeting nuclear DNA not only promotes successful quantification and genotyping of questioned samples with minute amounts of template DNA, but this approach also provides multiple target sites within the nuclear genome for designing multiplex assays that can simultaneously and species-specifically quantify DNA templates from a variety of species (Kanthaswamy et al. 2012).

We have developed a multiplex assay that utilizes the advantages of real-time quantitative PCR technology and nuclear genes to ensure efficient poultry species identification and accurate DNA quantification. Our assay uses species-specific hydrolysis probes and primers to target nuclear genes in common poultry animals including the T-cell surface glycoprotein *CD4* gene in duck (*Anas platyrhynchos*), the *transforming growth factor*, *beta 3* gene (*TGFB3*) in chicken (*Gallus gallus*), and the *prolactin receptor* gene (*PRLR*) in turkey (*Meleagris gallopavo*). The *TFGB3* and *PRLR* genes have previously been used in studies and have been determined to be species-specific (Köppel et al. 2009). While this assay is sensitive and specific to chicken, turkey and duck nuclear DNA detection and quantification, respectively, in single source samples, it is also capable of simultaneously detecting and quantifying all three poultry species' nuclear DNA in multi-species mixtures without cross-reactivity.

Materials and methods

DNA standards and samples

Purified and quantified high molecular weight genomic reference DNA extracts (Mallard (*Anas platyrhynchos*): Cat. # GD-220, Chicken: GC-120F, Turkey: GT-150) were purchased from Zyagen (San Diego, CA) and used as controls and quantification standards for all experiments. The remaining samples were used for the population study. Purified and quantified chicken DNA extracts (N=8) were obtained *ex gratia* from Scidera (Davis, CA). Duck feathers from ten individuals, each representing one of the following breeds: White Layer, Buff, Khaki Campgell, Welsh Harlequin, Cayuga, Black Runner, Mallard, Chocolate Runner, Blue Runner, and Black Swedish were obtained *ex graita* from Metzer Farms (Gonzales, CA). These duck breeds were derived from the wild Mallard (*Anas platyrhynchos*) which is the most commonly consumed species (Hird et al. 2005). Buccal swabs from livestock in the Davis, CA area were collected with owners' consent. The basal tip of the calamus and the blood clot from the superior umbilicus of the feathers were isolated following procedures by Horváth et al. (2005) and extracted and purified together using the QIAmp DNA Mini Kit (Qiagen Inc., Valencia, CA) according to the vendor's tissue extraction protocol. Buccal samples were extracted and purified using the same kit following the vendor's buccal extraction protocol. The concentrations of extracted samples were quantified with the Qubit™ fluorometer (Invitrogen, Carlsbad, CA) using the Quant-it™ dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA).

Primer and probe design

The chicken and turkey primers and probes were the same as those designed and published by Köppel et al. (2009). All duck nuclear genes and corresponding genes for chicken and turkey were downloaded from GenBank and aligned using Sequencher v.4.10.1 (GeneCodes, Corp., Ann Arbor, MI) to screen for duck specific sequences for *in silico* primer and probe design using Primer Express™ v.3.0 (Applied Biosystems, Carlsbad, CA). Candidate primer and probe sequences were screened *in silico* and in vitro following the same procedures as our previous work (Ng et al. 2012) for species specificity.

qPCR multiplex assay design

The qPCR assay was designed and optimized for a qPCR cocktail volume of 17 μL and 3 μL of DNA. Each reaction cocktail consisted of 12 μL of FastStart Universal Probe Master (ROX) 2X Concentration (Roche Diagnostic Corporation, Indianapolis, IN), primers and probes (FAM, VIC, NED dyes: Applied Biosystems, Carlsbad, CA) as listed in Table 1 and distilled deionized water for a total volume of 17 μL. Three microliters of DNA consisting of 10 ng/μL DNA for each of the three target poultry species was used in the multiplex. The qPCR run condition was the same as previously published (Ng et al. 2012).

Table 1 Oligonucleotide sequences and concentrations

Oligonucleotide	Final conc. (μM)	Sequence, 5′ - 3′	Position	Length	GenBank accession no.
Duck-F	0.2	CCAGTCCCATCCCACCTAATGTC	1067–1089	80	AY738732
Duck-R	0.2	CTGTTTTGACTTCTTGCCGTCCAT	1123–1146		
Duck-probe	0.1	**VIC**-GGTCCCATTAACACGTTCCCA-**MGB-NFQ**	1099–1119		
Chicken-F*	0.2	CAGCTGGCCTGCCGG	3323–3337	76	X60091
Chicken-R*	0.2	CCCAGTGGAATGTGGTATTCA	3378–3398		
Chicken-probe*	0.1	**FAM**-TCTGCCACTCCTCTGCACCCAGT **-MGB-NFQ**	3350–3372		
Turkey-F*	0.8	CAAAGAAAGCAGGGAAAAGGA	1799–1819	83	L76587
Turkey-R*	0.8	TGCACTCTCGTTGTTAAAAAGGA	1859–1881		
Turkey-probe*	0.2	**NED**-CTGGGAAAGTTACTGTGTAGCCTCAGAACG-**MGB-NFQ**	1821–1850		

*The primer and probe sequences for chicken and turkey were previously published by Köppel et al. (2009)

Probes are listed in the following format: **REPORTER DYE**-Sequence-**Minor Groove Binder – Non-Fluorescent Quencher**

Standard curves

Standard curves were generated and efficiencies calculated following the same procedures as previously published (Ng et al. 2012).

Validation studies

Validation studies were conducted using the same procedures as published previously in Ng et al. (2012). The dilution and inhibition studies were performed with 10 ng/μL of DNA from each target species for a total DNA concentration of 30 ng/μL. The only modification was in the non-target DNA dilution study. The non-target DNA species used in equal amounts for diluting included Bovine (Cat. # GB-110), Sheep (GS-190), Goat (GG-150), Pig (GP-160), Equine (GE-170), Cat (GC-130F), Dog (GD-150F), and Human (GH-180F) purchased from Zyagen (San Diego, CA). Since the chicken and turkey primers and probes were previously validated by Köppel et al. (2009) to be species-specific, a population study was not done for these two species. However, the duck primers and probe were designed in-house and were validated for specificity using the population study.

Results and discussion

Standard curves

Table 2 lists the calculated efficiency results from the standard curves for each target species. QPCR reactions have a maximum efficiency of 100 % since the amount of DNA can only be doubled per cycle. However, efficiencies greater than 100 % have been observed (Clark-Langone et al. 2010; da Silva Coelho et al. 2010), but are most likely due to pipetting errors and other reaction inefficiencies such as primer dimer. Figure 1 shows the species-specificity of the assay.

Validation studies

Dilution study

Serial dilution mixtures with equal amounts of chicken, duck, and turkey DNA samples were run to test the limit of detection (LOD) of the assay. The assay was able to detect all species down to $1{:}10^3$ dilutions (5 pg of each target species) which is less than the amount of nuclear DNA in a diploid cell (6 pg). In the non-target dilution study, all species were detected at $1{:}10^2$ dilutions with duck and turkey samples consistently detected down to $1{:}10^3$ dilutions.

Degradation study

DNA samples not treated with DNase showed very bright, large molecular weight bands consistent with non-degraded genomic DNA. The DNA mixtures treated with DNase consistently showed smeared bands for the 2.5 min time interval. All species were detected by qPCR for the digestion time intervals 0 and 5 min and only the chicken DNA sample was detected after 10 min's digestion.

Table 2 Standard curves and efficiencies

Species	Equation	R^2	Efficiency
Duck	$y = -3.3769x + 29.04$	0.9986	97.76 %
Chicken	$y = -3.3146x + 30.955$	0.9914	100.31 %
Turkey	$y = -3.0574x + 30.713$	0.996	112.36 %

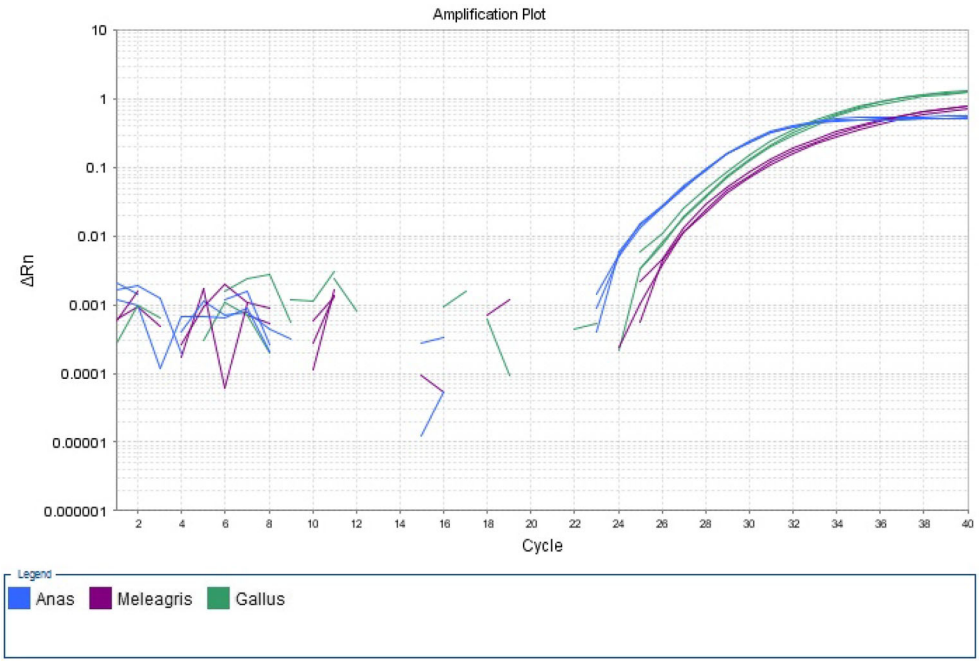

Fig. 1 Multiplex amplification plot showing species-specificity

Inhibition studies

Hematin completely inhibited the qPCR reactions for samples combined with 1 mM to 31.25 μM of inhibitor. Only the duck DNA sample was detected in 15.63 and 7.81 μM of hematin and all samples were detected at 3.91and 1.95 μM of hematin. Humic acid completely inhibited the qPCR reactions from 1000 ng/μL to 15.6 ng/μL of inhibitor. Only the duck sample was detected at 7.81 ng/μL of humic acid and duck and chicken samples were consistently detected at 3.91 ng/μL. All species were detected at 1.95 ng/μL of humic acid.

Population study

The duck assay was the only assay subjected to the population study. Each duck sample was run in duplicate and was the only sample detected in each reaction. The reactions did not show signs of inhibition for any of the individuals or any inconsistencies between individuals. Since all 10 individuals were reliably detected and correctly identified without any inter-individual differences, it is reasonable to postulate that the target primer and probe annealing sites do not have mutations that affect primer and probe binding and the target fragment does not have mutations that affect the efficiency of the reaction and species specificity.

Though few poultry assays have been developed for detecting food adulteration and fraudulence (Stamoulis et al. 2010), increasing consumer awareness of food adulteration has led to more assays involving poultry species. With poultry increasingly becoming the preferred meat of many individuals, poultry meats adulterated with other meats (Soares et al. 2010; Doosti et al. 2011; Stamoulis et al. 2010) have become a focus of study as opposed to poultry meats being the adulterant. In addition to consumer demands for food transparency, food fraudulence has severe economical effects on the food industry. The quick and accurate detection of poultry species with qPCR can reduce fraudulent replacement of higher quality meats with lesser quality meats and provide quantification data for subsequent individual identification and/or traceability testing.

In our study, we analyzed pure DNA extracts of three target poultry species. Since DNA is present in all tissues, we collected samples that were the least invasive to the animals even though our assay will most likely be applied to meat samples. The detection of our targets is based on the amount and quality of DNA in the reaction and not from which the DNA was derived. The quantitative analysis of heterogeneous meat samples (i.e. spiked meat) was considered, but the detection and quantification of targets in mixed meats is dependent on the sampling method, DNA extraction method, and assay detection. The mixed meat analysis was not done because there is no definitive method to determine if the assay is capable of accurately quantifying the DNA or if the variation in DNA profiles that are generated are caused by the differences in sampling or extraction methods.

Conclusion

We designed and developed a simple to use, one reaction step TaqMan®-based multiplex qPCR assay for the identification and DNA quantification of mixed poultry samples including duck, chicken, and turkey. Our assay has a limit of

detection of 5 pg of poultry DNA. In the presence of overwhelming non-target DNA, the LOD of the assay is 50 pg of DNA for chicken and 5 pg for duck and turkey DNA, respectively. In addition to its use in the food industry for safety and fraudulence management, the simplicity and sensitivity of this assay lends itself to be very useful in criminal or civil forensic investigations that involve poultry biological evidence. The assay was successful in the identification and quantification of the target species even in the presence of 3.91 μM of hematin and 1.95 ng/μL of humic acid, two PCR inhibitors known to be highly potent. Therefore, while our assay can contribute to the quality and safety assurance of our food supply through the facilitation of downstream STR- and SNP-based identity testing and traceability assays, our robust qPCR assay can similarly be applied to downstream analyses in the animal forensic science field such as evidentiary and reference sample comparisons.

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