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Methods

Quantitative PCR for DNA identification based on genome-specific interspersed repetitive elements

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Abstract

We have designed and evaluated a series of class-specific (Aves), order-specific (Rodentia), and species-specific (equine, canine, feline, rat, hamster, guinea pig, and rabbit) polymerase chain reaction (PCR)-based assays for the identification and quantitation of DNA using amplification of genome-specific short and long interspersed elements. Using SYBR Green-based detection, the minimum effective quantitation levels of the assays ranged from 0.1 ng to 0.1 pg of starting DNA template. Background cross-amplification with DNA templates derived from sixteen other species was negligible prior to 30 cycles of PCR. The species-specificity of the PCR amplicons was further demonstrated by the ability of the assays to accurately detect known quantities of species-specific DNA from mixed (complex) sources. The 10 assays reported here will help facilitate the sensitive detection and quantitation of common domestic animal and bird species DNA from complex biomaterials.

Keywords: Short interspersed elements; Long interspersed elements; Forensic genomics

The identification of biomaterials from complex sources is an important issue in both comparative genomics and investigative forensics. The ability to decipher each component of a multi-species DNA mixture is often essential to unraveling complicated aspects of a suspected crime scene or an environmental incident. Events such as earthquakes, tornadoes, and terrorist attacks create such volatile conditions, that the biological evidence available for postevent genomic analysis may include human and a nonhuman component. World events therefore dictate the need for reliable methods for the sensitive detection and quantitation of common domestic species from mixed sources.

Early approaches to identify species-specific components within mixed samples involved the use of high-performance liquid chromatography [1,2]. These methods have proven useful for the identification of many different animal species, but these approaches are labor intensive and the detection limits are restrictive. The detection of nuclear DNA sequences has also been useful in this regard, but are limited as a result of the single-copy nature of many of the sequences that are being assayed [3]. PCR analysis of species-specific mitochondrial DNA sequences is the most common method currently used for species identification [4–12]. The advantage of mitochondrial-based DNA analyses derives from the fact that there are many mitochondria per cell, and many mitochondrial DNA molecules within each mitochondrion making mitochondrial DNA a naturally amplified source of genetic variation. However, many of these systems require additional manipulations in the analysis such as restriction endonuclease digestion. Recently, PCR-based methods using multicopy nuclear DNA sequences such as satellite DNA [13,14] and repetitive elements [15–17] have been introduced. Like mitochondrial-based systems, these nuclear PCR-based assays take advantage of multiple target amplification sites in the genome of interest. For example, MS-PCR using MIR repeat elements [17] is specific for 12 different domestic and wild animal species. However, this method requires 10 h of electrophoresis using an automated sequencer to detect PCR products, and the results are

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not quantitative. In addition, the detection of unique species from mixed samples is problematic using this approach. Recently, the development of PCR assays based on sequences of short and long interspersed elements (SINEs and LINEs) has been reported for the detection of human DNA [18] as well as ruminant-, pig-, and chicken-derived materials [16]. Quantitative SINE-based PCR assays have been developed for the detection and quantitation of human DNA [19] and bovine, porcine, chicken and ruminant species DNA [20]. To overcome the limitations of existing PCR-based detection methods and to augment the assortment of quantitative SINE-based PCR assays currently available, we have developed a series of class-, order-, and species-specific assays based upon PCR amplification of short and long interspersed elements.

Results

Here, we report the development of a series of class-, order-, and species-specific assays for the identification and quantitation of DNA based upon intra-SINE/LINE PCR amplification of genome-specific interspersed repetitive elements (Tables 1 and 2). Interspersed elements reside within almost every genome that has been studied to date [21,22]. Most mammalian SINEs have amplified in the past 65 million years and are thought to have been spread throughout each genome via an RNA-mediated duplication process termed retroposition [21]. Because most of the SINE families within different genomes were derived independently, every mammalian order has a significant number (in excess of 100,000) of characteristic mobile elements. These large dispersed gene families serve as novel markers that identify the DNA from the species within that order, thus providing specific genomic tags that can be used in conjunction with PCR to amplify specific subsets of genomic sequences unique to the genome or species of interest from mixed DNA sources. During intra-element PCR amplification, oligonucleotide primers are designed within the core body of the element consensus sequence to amplify multiple target sites of the element and generate a homogeneous product composed entirely of the repeat core unit DNA sequences characteristic of the genome being amplified.

Using conventional PCR and the conditions shown in Table 2, assays for detection of equine, canine, feline, rat, hamster, guinea pig, and rabbit DNA were shown to be species-specific (Figs. 1A–1D and 1F–1H). The mouse assay based on the B4/SINE repeat produced a PCR amplicon from rat, mouse, and hamster DNA, members of the Rodentia order, but not from guinea pig DNA (Fig. 1E). The assay based on the Anseriforme order [23], or "water bird" genome produced a PCR amplicon from duck (*Anas discors*), but also from dove (*Columbina passerina*) DNA templates that are not considered to be water birds (Fig. 1J). However, this assay did not amplify chicken (*Gallus gallus*) templates, in contrast to the class-specific avian assay (Figs. 1I and 3I) and the species-specific chicken intra-CR1 SINE-based PCR assay [20].

Using quantitative PCR with SYBR green fluorescence detection, the equine intra-ERE-1 SINE-based PCR assay had a linear quantitation range of 10–0.00001 ng (0.01 pg), or 10^7, as shown by the standard curve (Fig. 2A). The mean value of the no template control (NTC) was 34.1 ± 0.5 and significantly different from 31.2 ± 0.6 at the 0.01 pg level ($p = 0.0131$). This assay detected the known values of equine DNA within mixed-DNA samples from 50% (5 ng) to 0.05% (5 pg) as indicated by the open triangles on the standard curve. The compositions of the various DNA mixtures are shown in Table 3. A total of 10 ng of DNA template was used in each test. Background cross-amplification was detected in bovine (*Bos taurus*) and ovine (*Ovis aries*) DNA templates following 29 cycles of PCR when tested with an equivalent amount of DNA (2 ng) (Fig. 3A). Therefore, cross-species amplification limits the effective quantitation range of this equine intra-SINE PCR assay to 0.1 pg (0.001% in 10 ng starting template) when equivalent amounts of cow or sheep DNA may be present in the samples. The sensitivity of this quantitative range is attributable to the estimated 20,000–80,000 copies of the ERE-1 SINE present in the equine genome [24,25].

The canine intra-SINE-based PCR assay had a linear quantitation range of 100–0.0001 ng (0.1 pg), or 10^7 (Fig.

Table 1
Repetitive elements for intra-SINE/LINE PCR detection assays

Name	Class	Order	Family	Genus and species	Repeat element	Accession no.
Equine	Mammalia	Perissodactyla	Equidae	*Equus caballus*	Ere-1 SINE/horse	D26566
Canine	Mammalia	Carnivora	Canidae	*Canis familiaris*	SINEC_CF SINE/dog	X57357
Feline	Mammalia	Carnivora	Felidae	*Felis catus*	B2_Mv SINE/carnivores	AC090033
Rat	Mammalia	Rodentia	Muridae	*Rattus norvegicus*	L1_RN LINE/L1	AC087102
Mouse	Mammalia	Rodentia	Muridae	*Mus musculus*	RSINE1 SINE/B4	AC012526
Hamster	Mammalia	Rodentia	Muridae	*Cricetulus griseus*	B2_Mm2 SINE/B2	X96664
Guinea pig	Mammalia	Rodentia	Caviidae	*Cavia procellus*	ID3 SINE/ID	AF312680
Rabbit	Mammalia	Lagomorpha	Leporidae	*Lepus*	C_Oc SINE/rabbit	Y00347
Avian	Aves	N/A	N/A	N/A	L3b LINE/CR1	AC092403
Water bird	Aves	Anseriformes	Anatidae	N/A	L3b LINE/CR1	X57379

Table 2
Oligonucleotide primers and amplification conditions for intra-SINE/LINE PCR detection assays

Name	5′ Primer sequence (5′–3′)	3′ Primer sequence (5′–3′)	AT	Size (bp)	[$MgCl_2$]
Equine	GTTTCGTTGGTTCGAATCCTG	ATTCTTCATTGTGGGTCCTTCT	65	105	1.1 mM
Canine	AGGGCGCGATCCTGGAGAC	AGACACAGGCAGAGGGAGAA	55	83	0.9 mM
Feline	AGTCGGTTAAGCGTCTGACTTT	CTCCAGGCTCTGAGCTGTCA	55	98	1.1 mM
Rat	CAAGACGGATGATCAAAATGTG	ATTGGGTGGCTGTATATGTATGG	61	161	0.9 mM
Mouse	AGATGGCTCAGTGGGTAAAGG	GTGGAGGTCAGAGGACAAACTT	55	118	1.5 mM
Hamster	GCTCAGAGGTTAAGAGCACTGAC	TGCTTCCATGTATATCTGCACAC	60	132	1.0 mM
Guinea pig	GGGATTTAGCTCAGTGGCATAAG	ATTGGTACCGGGGATTGAACT	60	71	1.1 mM
Rabbit	TGGTTCACTCCCCAAATACCT	CCACAGCTCCACTTCTGATCTA	60	160	1.5 mM
Avian	ATAGAATGGCCTGGGTTGAAAAG	AAGTTTTTCACACAGAGGGTGGT	55	197	1.5 mM
Water bird	TAGCGAGGTGGGTATTGGTC	GGATGGTGACTCCACCACTT	65	174	3.0 mM

2B), and the NTC was 36.5 ± 1.0, not significantly different from 33.4 ± 0.1 at the 0.1 pg level ($p = 0.1552$). This assay detected the known values of canine DNA within mixed-DNA samples from 50% (5 ng) to 0.05% (5 pg) (Fig. 2B) and background cross-amplification was negligible (Fig. 3B). Therefore, the minimum effective quantitation level

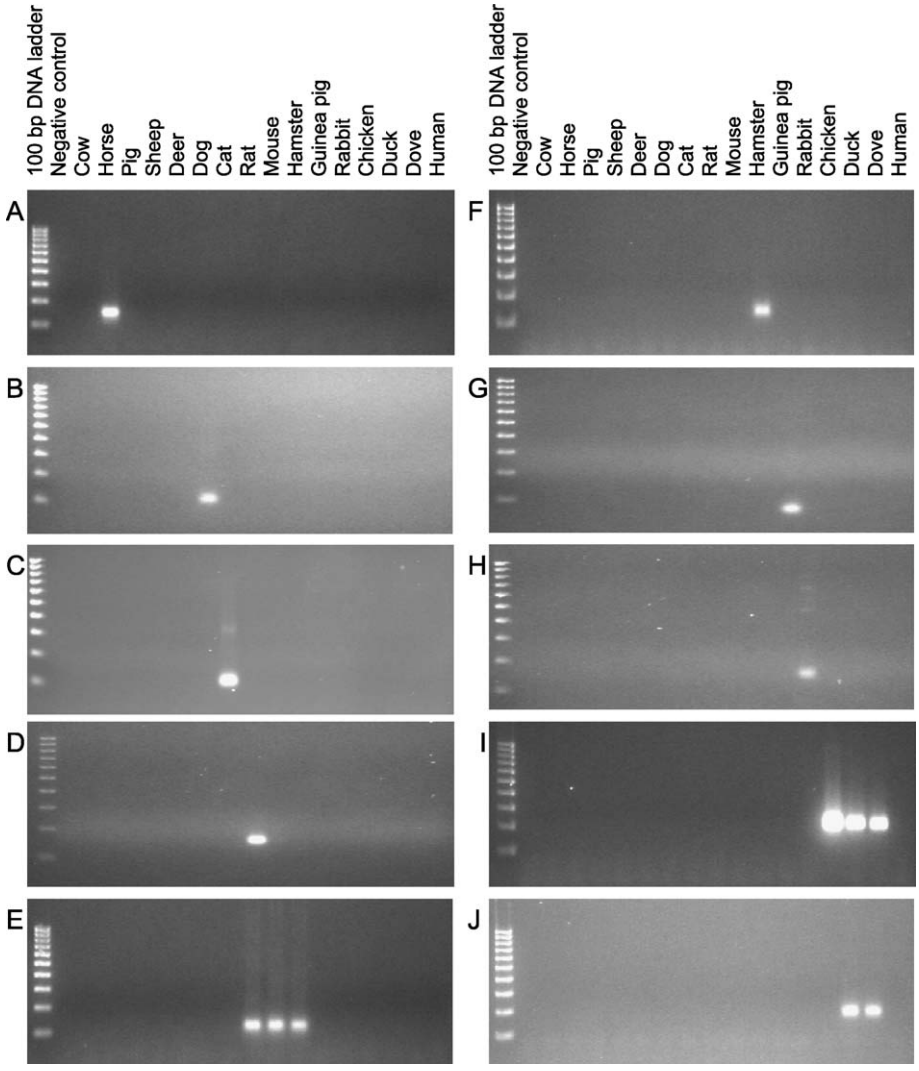

Fig. 1. Species-specific DNA detection using 10 intra-SINE/LINE-based assays. The results following 30 cycles of conventional PCR using (A) the equine-specific assay, (B) the canine-specific assay, (C) the feline-specific assay, (D) the rat-specific assay, (E) the Rodentia order-specific assay designed in the mouse B4/SINE repeat, (F) the hamster-specific assay, (G) the guinea pig-specific assay, (H) the rabbit-specific assay, (I) the class-specific avian assay, or (J) the Anseriforme ("water bird") order-based assay are shown. Amplicons from the assays were chromatographed on a 2% agarose gel that contained ethidium bromide and products visualized using UV fluorescence.

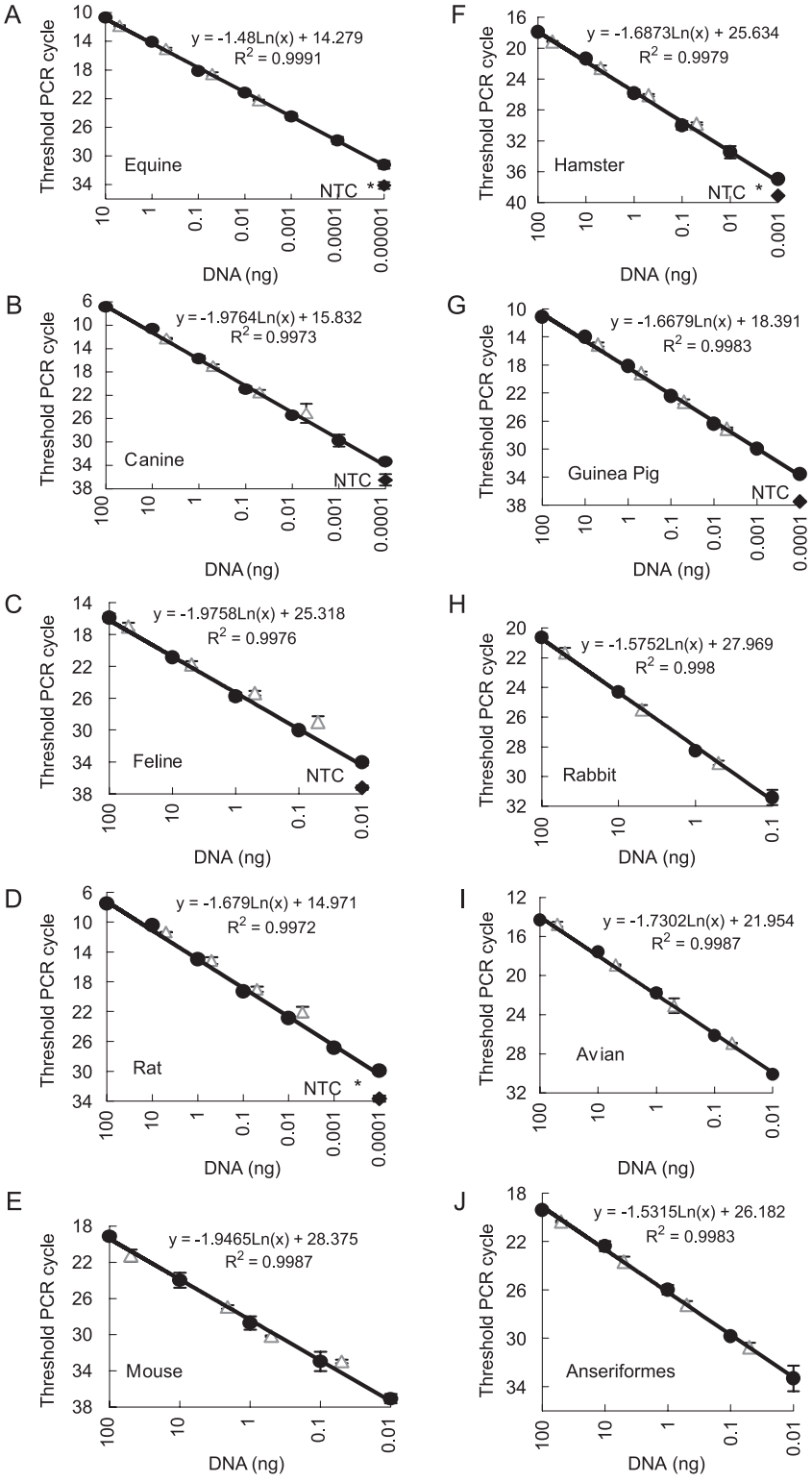

Fig. 2. Quantitation range for 10 intra-SINE/LINE-based assays. The effective quantitation ranges for assays A–J using SYBR green fluorescence detection are shown. The PCR cycle at which the fluorescent signal crosses baseline is considered to be the threshold cycle, plotted on the *y* axis. The fluorescent signal produced by a 10-fold dilution series of (A) horse, (B) dog, (C) cat, (D) rat, (E) mouse, (F) hamster, (G) guinea pig, (H) rabbit, (I) chicken, or (J) duck DNA is plotted as the mean of duplicates ± 1 standard deviation. The R^2 value is 99–100% for all standard curves. An asterisk (*) by the NTC (no template control) indicates that the NTC value was significantly ($p \leq 0.05$) different from the final point on the standard curve. Analyses of the various species DNA mixtures outlined in Table 3 are plotted as open triangles along the appropriate standard curve as the mean of duplicates ± 1 standard deviation. Their alignment along the standard curve demonstrates the utility of each assay to quantitate a DNA of interest from within a complex source of starting templates.

Table 3
Compositions of mixed-species DNA test samples

Contents	Equine	Porcine	Human	Total template
	DNA (ng) (%)	DNA (ng) (%)	DNA (ng) (%)	DNA (ng) (%)
Equine mix				
1	5 (50)	5 (50)	0 (0)	10 (100)
2	0.5 (5)	5 (50)	4.5 (45)	10 (100)
3	0.05 (0.5)	5 (50)	4.95 (49.5)	10 (100)
4	0.005 (0.05)	5 (50)	4.995 (49.95)	10 (100)
	Canine	Feline	Human	Total template
Canine mix				
1	5 (50)	5 (50)	0 (0)	10 (100)
2	0.5 (5)	5 (50)	4.5 (45)	10 (100)
3	0.05 (0.5)	5 (50)	4.95 (49.5)	10 (100)
4	0.005 (0.05)	5 (50)	4.995 (49.95)	10 (100)
	Feline	Canine	Human	Total template
Feline mix				
1	50 (50)	25 (25)	25 (25)	100 (100)
2	5 (50)	2.5 (25)	2.5 (25)	10 (100)
3	0.5 (5)	5.0 (50)	4.5 (4.5)	10 (100)
4	0.05 (0.5)	5.0 (50)	4.95 (49.5)	10 (100)
	Rat	Hamster	Mouse	Total template
Rat mix				
1	5 (50)	5 (50)	0 (0)	10 (100)
2	0.5 (5)	5 (50)	4.5 (45)	10 (100)
3	0.05 (0.5)	5 (50)	4.95 (49.5)	10 (100)
4	0.005 (0.05)	5 (50)	4.995 (49.95)	10 (100)
	Mouse	Guinea pig	Human	Total template
Mouse mix				
1	50 (50)	25 (25)	25 (25)	100 (100)
2	5 (50)	2.5 (25)	2.5 (25)	10 (100)
3	0.5 (5)	5.0 (50)	4.5 (4.5)	10 (100)
4	0.05 (0.5)	5.0 (50)	4.95 (49.5)	10 (100)
	Hamster	Guinea pig	Human	Total template
Hamster mix				
1	50 (50)	25 (25)	25 (25)	100 (100)
2	5 (50)	2.5 (25)	2.5 (25)	10 (100)
3	0.5 (5)	5.0 (50)	4.5 (4.5)	10 (100)
4	0.05 (0.5)	5.0 (50)	4.95 (49.5)	10 (100)
	Guinea pig	Rat	Mouse	Total template
Guinea pig				
1	5 (50)	5 (50)	0 (0)	10 (100)
2	0.5 (5)	5 (50)	4.5 (45)	10 (100)
3	0.05 (0.5)	5 (50)	4.95 (49.5)	10 (100)
4	0.005 (0.05)	5 (50)	4.995 (49.95)	10 (100)
	Rabbit	Rat	Mouse	Total template
Rabbit mix				
1	50 (50)	25 (25)	25 (25)	100 (100)
2	5 (50)	2.5 (25)	2.5 (25)	10 (100)
3	0.5 (5)	5.0 (50)	4.5 (4.5)	10 (100)
4	0.05 (0.5)	5.0 (50)	4.95 (49.5)	10 (100)
	Chicken	Bovine	Human	Total template
	DNA (ng) (%)	DNA (ng) (%)	DNA (ng) (%)	DNA (ng) (%)
Avian mix				
1	50 (50)	25 (25)	25 (25)	100 (100)
2	5 (50)	2.5 (25)	2.5 (25)	10 (100)
3	0.5 (5)	5.0 (50)	4.5 (4.5)	10 (100)
4	0.05 (0.5)	5.0 (50)	4.95 (49.5)	10 (100)
	Duck	Chicken	Human	Total template
Duck mix				
1	50 (50)	25 (25)	25 (25)	100 (100)
2	5 (50)	2.5 (25)	2.5 (25)	10 (100)
3	0.5 (5)	5.0 (50)	4.5 (4.5)	10 (100)
4	0.05 (0.5)	5.0 (50)	4.95 (49.5)	10 (100)

of this assay is 1 pg (0.01%) when testing DNA samples from complex (mixed) sources. The canine SINE used in this assay is thought to occupy 1.8–3% of the canine genome [26].

The feline intra-SINE-based PCR assay had a linear quantitation range of 100–0.01 ng, or 10^5 (Fig. 2C) and the NTC was 37.2 ± 0.3, not significantly different from 36.9 ± 0.8 at the 0.01 ng level ($p = 0.5269$). This assay detected known amounts of feline DNA at the 50% level (50 and 5ng) within mixed-DNA samples of feline, canine, and human DNA, but overestimated the feline portion of DNA in the mixtures at lower levels. This is illustrated by the open triangles on the standard curve at the 50- and 5-ng levels, but trailing above the standard curve at the 5 and 0.5% feline DNA levels (Fig. 2C). This limits the effective quantitation range of this feline intra-SINE-based PCR assay to about 1 ng (10%) when testing DNA samples composed of multiple species of the Carnivora order. There are an estimated 200,000 copies of the carnivore SINEs known as CAN-SINEs in the feline genome [27]. However, using the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) BLAST function and "limit by entrez query" tool, a search of the feline genome for the nucleotide sequence used in this assay (98 bp), resulted in approximately 1000 matches.

The rat intra-LINE-based PCR assay had a linear quantitation range of 100–0.0001 ng (0.1 pg), or 10^7 (Fig. 2D), and the NTC was 33.7 ± 0.4, significantly different from 30.0 ± 0.4 at the 0.1 pg level ($p = 0.0051$). This assay detected the known values of rat DNA within mixed-rodent DNA samples from 50% (5 ng) to 0.5% (50 pg), but slightly overestimated the rat portion of the rodent DNA mixture at the 0.05% level, as illustrated by the open triangles on the standard curve at the 50, 5, and 0.5% levels, but trailing above the curve at the 0.05% rat DNA level (Fig. 2D). Therefore, the minimum effective quantitation level of this rat intra-LINE-based assay is 0.1 ng (1%) when testing DNA samples composed of multiple species of the Rodentia order. A BLAST search of the rat genome using the 161-bp intra-LINE nucleotide sequence used in this assay resulted in about 70,000 matches.

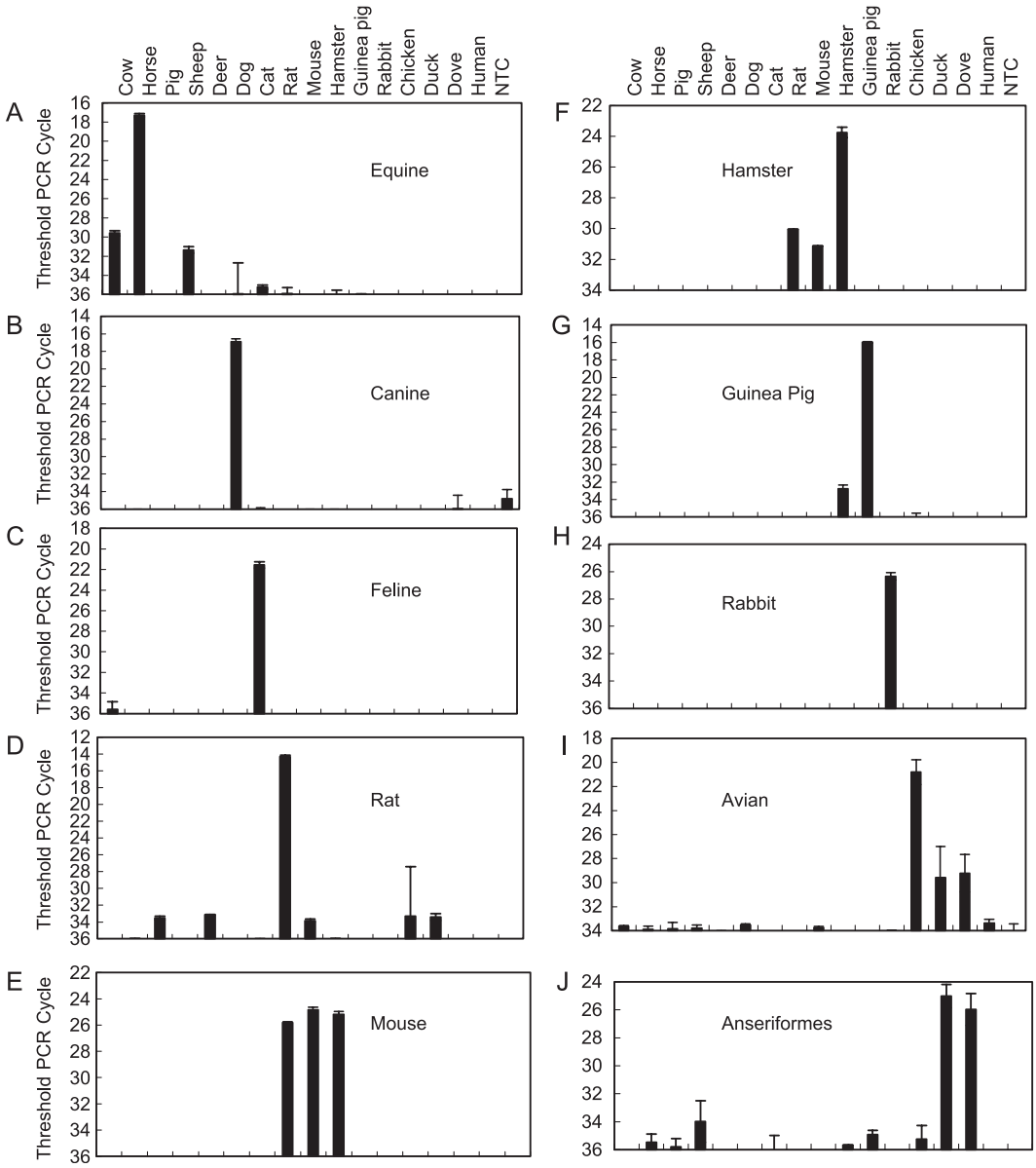

Fig. 3. Background PCR amplification using DNA templates from 16 species. The cross-amplification of DNA templates derived from various species is shown for intra-SINE/LINE-based assays A–J using SYBR green fluorescence detection. The PCR cycle at which the fluorescent signal crosses baseline is considered to be the threshold cycle, plotted on the *y* axis (mean of duplicates ± 1 standard deviation). Using DNA (2 ng) from 16 different species as template, background amplification was detected in trace amounts in the equine-, rat-, hamster-, guinea pig-, and Anseriforme-based assays following 30 cycles of PCR.

The intra-B4-SINE PCR assay designed using mouse genomic sequence, also identified rat and hamster DNA of the Rodentia order with equal efficiency (Figs. 1E and 3E), making this assay order-specific rather than species-specific. Using mouse DNA template this assay had a linear quantitation range of 100–0.01 ng, or 10^5 (Fig. 2E) and the NTC was not detectable. This assay identified the known values of mouse DNA within mixed-DNA samples of mouse, guinea pig, and human DNA from 50% (50 and 5 ng) to 5% (0.5 ng) but slightly overestimated the mouse portion of the DNA mixture at the 0.5% (50 pg) level as indicated by the open triangles. Therefore, the minimum effective quantitation level of this assay is about 0.1 ng (1%) during detection of rat, mouse, or hamster DNA from mixed sources. A BLAST search of the mouse genome using the 118-bp intra-B4-SINE nucleotide sequence used in this assay resulted in about 2000 hits.

The hamster intra-B2-SINE PCR assay had a linear quantitation range of 100–0.001 ng (1 pg), or 10^6 (Fig. 2F), and the NTC was 39.1 ± 0.0, significantly different from 36.9 ± 0.0 at the 1 pg level ($p = 0.0060$). This assay detected the known values of hamster DNA within mixed-

DNA samples of hamster, guinea pig, and human DNA from 50% (50 and 5 ng) to 0.5% (50 pg) (Fig. 2F). However, background cross-amplification was detected in both rat (*Rattus norvegicus*) and mouse (*Mus musculus*) DNA templates following 30 cycles of PCR when tested with an equivalent amount of DNA (2 ng) (Fig. 3F). Therefore, the minimum effective quantitation level of this intra-B2-SINE hamster assay is restricted to about 0.1 ng (1%) (threshold PCR cycle 30) when testing DNA samples composed of multiple rodent species. Although the B2-SINE family in general constitutes about 0.7% of the mouse genome [28], a BLAST search of the hamster genome using our 132-bp PCR amplicon designed from hamster sequence [29] resulted in only about 100 matches.

The guinea pig intra-SINE-based PCR assay had a linear quantitation range of 100–0.0001 ng (0.1 pg), or 10^7 (Fig. 2G), and the NTC was 37.5 ± 0.1, not significantly different from 33.6 ± 0.4 at the 0.1 pg level ($p = 0.0564$). This assay detected the known values of guinea pig DNA within mixed-DNA samples of guinea pig, rat, and mouse DNA from 50% (5 ng) to 0.05% (5 pg) (Fig. 2G) and background cross-amplification was negligible, even among other species of the Rodentia order (Fig. 3G). Therefore, the minimum effective quantitation level of this assay is 1 pg (0.01%) when testing DNA samples from mixed sources. The guinea pig ID-SINE used in this assay has an estimated 200–3000 copies in the guinea pig genome [30].

The rabbit intra-SINE-based PCR assay had a linear quantitation range of 100–0.1 ng (Fig. 2H) and the NTC was not detectable. This assay detected the known values of rabbit DNA within mixed-DNA samples from 50% (50 and 5 ng) to 5% (0.5 ng) and background cross-amplification was negligible (Fig. 3H). Therefore, the minimum effective quantitation level of this assay is 0.1 ng (1%) when testing DNA samples from mixed sources. A BLAST search of the rabbit genome for the rabbit nucleotide sequence [31] used in this assay (160 bp) resulted in about 170 matches.

The class-specific avian intra-CR1-LINE-based PCR assay detected all bird species tested (Fig. 1I) but not with equal efficiency (Fig. 3I). Using chicken DNA template this assay had a linear quantitation range of 100–0.01 ng, or 10^5 (Fig. 2I), and the NTC was 37.0 ± 0.0, significantly different from 30.1 ± 0.2 at the 0.01 ng level ($p = 0.0139$). This assay detected the known values of chicken DNA within mixed-DNA samples of chicken, bovine, and human DNA from 50% (50 and 5 ng) to 0.5% (50 pg), and background cross-amplification with nonavian DNA was negligible (Fig. 3I), indicating that the minimum effective quantitation level of this assay is 0.01 ng (0.1%) when testing DNA samples from mixed sources. However, the unequal detection affinity for avian DNA among various species means that DNA standards for the avian species of interest would be required for accurate quantitative analyses. Although, this somewhat restricts the utility of the assay for quantitative analyses, it does not compromise the ability of the assay to detect multiple species of the Aves class simultaneously. A BLAST search of the Aves genome using the 197-bp intra-CR1-LINE ampicon used in this assay resulted in about 350 matches.

The intra-LINE-based PCR assay designed using DNA sequence from the Anseriforme order [23], had a linear quantitation range of 100–0.01 ng, or 10^5 (Fig. 2J), and the NTC was not detectable. This assay detected known amounts of duck (*Anas discors*) DNA within mixed-DNA samples of duck, chicken, and human DNA from 50% (50 and 5 ng) to 0.5% (50 pg). However, dove (*Columbina passerina*) DNA was also detected with equal efficiency (Figs. 1J and 3J). Therefore, the minimum effective quantitation level of this assay is 0.01 ng when testing DNA samples from most complex sources. However, the affinity of this assay for dove DNA as well as water bird DNA limits it to being a class-specific (Aves) assay even though it exhibits no background cross-amplification affinity for chicken DNA.

Discussion

In this study we have designed and evaluated a series of class-specific (Aves), order-specific (Rodentia), and species-specific (equine, canine, feline, rat, hamster, guinea pig, and rabbit) PCR assays for the identification and quantitation of DNA from complex (mixed) sources. These assays are designed to augment the assortment of quantitative intra-SINE-based PCR assays recently reported for the identification and quantitation of human [19], bovine, porcine, chicken, and ruminant species DNA [20]. The sensitive and reliable identification of biomaterials from complex sources has long been an important objective in forensic science genomics. Several methods have been reported previously for species identification, including recent PCR-based approaches using repetitive elements [15–18]. However, there are several advantages to our intra-SINE/LINE-based PCR methods over previously reported approaches. First, these PCR assays do not require any additional processing steps such as restriction endonuclease digestion for analysis [9,11]. In addition, no special expertise or unique equipment, such as an automated DNA sequencer, is required [17]. Species-specific DNA detection can be performed by simple agarose gel analysis as an initial screening tool. This assay format minimizes the cost of performing these analyses on a large-scale and gives most laboratories with average resources the ability to perform these assays.

An additional advantage of our intra-SINE/LINE-based PCR assays over many previously reported detection methods is that these assays employ a nuclear sequence with a high copy number for amplification, while simultaneously maintaining some of the same advantages of single-locus PCR. For example, these amplicons are relatively short (Table 2) to minimize sensitivity to degraded DNA templates. In addition, the products of each assay are

uniform-size amplicons, making them amenable to multiple visualization and detection schemes such as ethidium bromide and UV fluorescence and SYBR green or TaqMan chemistry for quantitative PCR.

The use of SYBR green-based detection in the amplification protocol facilitates accurate quantitation using any quantitative PCR system. This represents a distinct advantage over species identification assays that are not quantitative [11,16,17]. In fact, most PCR-based identification assays currently available for the species reported here do not report a detection limit or other sensitivity data. Parodi, et al. [12], using PCR-based assays for species identification of animal cell lines, reported the detection limit to be about 800 cells for mouse, rat, hamster, dog, cat, rabbit and horse cell lines. Using *C*-value estimates, this corresponds to roughly 4 μg of DNA [32]. The detection limits of our intra-SINE/LINE-based quantitative PCR assays exceed this by several orders of magnitude.

We have also systematically evaluated the specificity of our quantitative intra-SINE-based PCR assays with regard to class, order, and species. In addition to agarose gel-based analysis following conventional PCR, we also used SYBR green-based fluorescence detection of amplified DNA from 16 different species to assess possible cross-species amplification. Some loss of species specificity was observed in the equine and hamster assays using SYBR green-based fluorescence detection as compared to conventional PCR, as evidenced by the presence of background signal (Figs. 3A and 3F, respectively), but no false banding indicated with conventional PCR (Figs. 1A and 1F, respectively). We attribute this to differences in $MgCl_2$ concentration between the two PCR master-mix formats. During SYBR green-based experiments, the $MgCl_2$ concentration was 3.0 mM for all reactions, in accordance with the recommendations of the manufacturer. But during the conventional PCR experiments $MgCl_2$ concentrations were optimized for each assay (Table 2). Although this must be taken into consideration when performing the equine or hamster assays to analyze complex samples quantitatively, the increase in background cross-species amplification occurred after 29 cycles of PCR (Fig. 3) and has been accounted for in the reported minimum effective quantitation levels (0.1 pg and 0.1 ng, respectively).

Assay specificity was further evaluated by the ability of the assays to detect accurately known trace quantities of species-specific DNA from mixed-species templates. The equine, canine, hamster, guinea pig, and rabbit species-specific assays effectively quantified the DNA component of interest from within a 10-ng DNA sample of multi-species templates, at levels ranging from 50 to 0.001%, 0.01, 1, 0.01, and 1%, respectively. Parodi et al. [12], reported detectable PCR amplification down to 1% from cells of interest within contaminating cells. However, these results were not quantitative and further demonstrate the unique properties of our intra-SINE/LINE-based quantitative PCR assays for these domestic species.

The class-specific (Aves) assays have limited utility for quantitative analyses of mixed avian species DNA. However, the collective use of these Aves-based assays serves several functions. First, the avian assay simultaneously detects all bird species. Second, the Anseriforme assay detects duck DNA and not chicken DNA. When used in conjunction with the species-specific chicken intra-CR1-SINE-based PCR assay [20] these Aves-based assays permit rapid screening for chicken, duck, and other bird species.

Interestingly, the feline, rat, and mouse assays tended to overestimate the component DNA of interest when trace quantities (5, 0.05, and 0.5%, respectively) were present in a multi-species DNA mixture. Yet, nonspecific amplification using the same DNA templates individually (Fig. 3) was negligible for all other species tested in the feline assay, for all other rodent species in the rat assay, and for guinea pig DNA in the mouse assay. One possible explanation for this phenomenon might be that the PCR primers used in these assays do not share sufficient sequence identity with the other species tested to form an amplicon from single-species templates, but when genomic DNA of multiple species is mixed together prior to PCR, there is sufficient homology among the genomes represented in the mixture to facilitate recombination or template switches during the denature/annealing steps of PCR cycling, thus creating new ‘‘mixed templates’’ with a sufficient number of complementary primer sites to create background amplification. Although this ‘‘noise’’ must be taken into consideration when performing these quantitative assays to analyze complex samples, the increase in background cross-species amplification occurred when attempting to detect only trace amounts of the species of interest within a multispecies DNA mixture. Furthermore, SYBR green binds to all double-stranded DNA, producing signal, whereas the use of sequence-specific probes (i.e., TaqMan probes) would likely eliminate this nonspecific background signal. Although, we did not evaluate the use of TaqMan-based detection with the assays reported here, the fact that each assay produces a uniform-size amplicon makes them amenable to multicolor multiplex detection for large-scale assay applications in the future.

Here, we have demonstrated the sensitivity and specificity of 10 class-specific (Aves), order-specific (Rodentia), and species-specific (equine, canine, feline, rat, hamster, guinea pig, and rabbit) quantitative PCR assays based on interspersed elements. The high copy number of SINEs/LINEs in various genomes makes these assays ideal for quantitative species-specific DNA detection. These assays possess the unique ability to analyze quantitatively multiple components of a multispecies DNA mixture when performed simultaneously. The multispecies DNA mixtures tested in these experiments contained various amounts of bovine, porcine, and human DNA in addition to the species of interest, further demonstrating the utility of these assays in combination with intra-SINE-based quantitative PCR assays reported previously [19,20]. Collectively, they provide an assemblage of

simple, reliable DNA-based tests for the sensitive detection and quantitation of human and nonhuman domestic species of animals and birds from complex biomaterials.

Materials and methods

Primer design and PCR amplification

The National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) "TaxBrowser" function was used to search for nucleotide sequences from various genomes of interest. DNA sequences were then subjected to computational analysis using the RepeatMasker server at the University of Washington (Seattle, WA, USA) (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker) to identify SINEs/LINEs contained within those genomes. Oligonucleotide PCR primers were designed using either Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) or Primer Express software (Applied Biosystems, Inc.) and purchased from MWG Biotech, Inc., or Sigma–Genosys, Inc. Each primer pair was evaluated in our laboratory for specificity and sensitivity using standard PCR and agarose gel electrophoresis. Only those oligonucleotide pairs meeting the project criteria were selected for further analysis (Tables 1 and 2). The SYBR green PCR core reagent kit was purchased from Applied Biosystems, Inc. (SYBR is a registered trademark of Molecular Probes, Inc.).

PCR conditions were optimized for each assay with regard to annealing temperature and concentration of $MgCl_2$. Conventional PCR reactions for agarose gel-based detection were carried out in 25 μl using 2 ng of DNA template, 1× PCR buffer II (Applied Biosystems, Inc.), 0.2 mM dNTPs, 200 nM each oligonucleotide primer, optimized $MgCl_2$ (Table 2), and 1 unit *Taq* DNA polymerase. Each sample was subjected to an initial denaturation of 1 minute at 95°C, followed by 30 amplification cycles of denaturation at 95°C for 30 s, optimized annealing (Table 2) for 30 s, followed by extension at 72°C for 30 s. Quantitative PCR reactions were carried out in 50 μl using 1 using 1×SYBR green buffer, 1 mM dNTPs, 3.0 mM $MgCl_2$ and 1.25 units AmpliTaq Gold DNA polymerase as recommended by the supplier. Each sample was subjected to an initial denaturation of 12 minutes at 95°C to activate the AmpliTaq Gold, followed by 40 amplification cycles of denaturation at 95°C for 20 s and 1 min of anneal/extension at either 60°C (rat, hamster, guinea pig, and rabbit) or 65°C (equine and water birds). Some assays required an intermediate annealing step at 55°C for 45 s, followed by extension at 60°C for 30 s (canine, feline, mouse, and avian). Each quantitative PCR reaction contained 49 μl of PCR master mix and 1 μl of DNA template. Quantitative PCR experiments were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Inc.)

DNA samples

DNA from cow (*Bos taurus*), horse (*Equus caballus*), sheep (*Ovis aries*), dog (*Canis familiaris*), cat (*Felis catus*), hamster (*Cricetulus griseus*), guinea pig (*Cavia procellus*), rabbit (*Oryctolagus cuniculus*), and dove (*Columbina passerina*) were obtained by tissue and blood extraction using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) and samples provided by the Louisiana State University School of Veterinary Medicine. Chicken (*G. gallus*) DNA was extracted from blood using the QIAamp DNA blood mini kit (QIAGEN, Inc., Valencia, CA, USA). DNA from pig (*Sus scrofa*), deer (*Odocoileus virginianus*), duck (*Anas discors*), rat (*Rattus norvegicus*), and mouse (*Mus musculus*) were prepared from tissue with proteinase K digestion followed by phenol:-chloroform extraction and ethanol precipitation [33]. Human DNA (HeLa cell line ATCC CCL2; American Type Culture Collection, Manassas, VA) isolations were performed using Wizard genomic DNA purification (Promega Corporation, Madison, WI). Extracted DNA was stored in 10 mM Tris/0.1 mM EDTA (TLE), quantified spectrophotometrically and then serially diluted 10-fold in TLE such that concentrations from 100 ng to 0.01 pg were assayed in duplicate using PCR.

Data analysis

Data from duplicate DNA standards were exported from the ABI Prism 7000 SDS software into a Microsoft Excel spreadsheet where the mean value and standard deviation were calculated for each point on the standard curve. Using the Excel trendline option, a line of best fit was plotted with y error bars equal to 1 standard deviation to form a standard curve. Data from the negative control (no template control-NTC) duplicates and the composite mixed-species DNA test samples (Table 3) (means ± 1 standard deviation of duplicates) were then plotted on the graph for comparison to the standard curve. Pairwise t tests were performed to determine if the NTC for each assay was statistically different from the minimum value on the standard curve ($p \leq 0.05$).

Data from the multispecies cross-amplification experiments were exported to Excel in a similar manner and the mean and standard deviation of duplicates were calculated. The Excel chart wizard was used to construct bar graphs with Y-error bars equal to 1 standard deviation.

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