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REVIEW

DNA detective: a review of molecular approaches to wildlife forensics

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Abstract Illegal trade of wildlife is growing internationally and is worth more than USD$20 billion per year. DNA technologies are well suited to detect and provide evidence for cases of illicit wildlife trade yet many of the methods have not been verified for forensic applications and the diverse range of methods employed can be confusing for forensic practitioners. In this review, we describe the various genetic techniques used to provide evidence for wildlife cases and thereby exhibit the diversity of forensic questions that can be addressed using currently available genetic technologies. We emphasise that the genetic technologies to provide evidence for wildlife cases are already available, but that the research underpinning their use in forensics is lacking. Finally we advocate and encourage greater collaboration of forensic scientists with conservation geneticists to develop research programs for phylogenetic, phylogeography and population genetics studies to jointly benefit conservation and management of traded species and to provide a scientific basis for the development of forensic methods for the regulation and policing of wildlife trade.

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International wildlife trade and forensic genetics

According to Interpol (International Policing Organisation), the illegal trade of plants, animals and their by-products is a growing global black market commerce estimated to be worth more than USD $20 billion per year [1]. Organised international criminal networks have been linked to the trafficking of wildlife using their established drug smuggling routes to illegally transport wildlife across international borders [2, 3]. In Brazil, recent estimates suggest that at least 40% of all illegal drugs shipments are combined with wildlife [4]. Similarly, one-third of all cocaine seized in 1993 was reported by the United States Fisheries and Wildlife Service (USFWS) to be associated with wildlife imports. The illicit wildlife trade is attractive to criminals because weight-for-weight wildlife is equally or more profitable than drugs or arms and with less associated risk. The rate of detection is lower and the penalties, if offenders are caught and convicted, are typically far more lenient for wildlife crimes than for drugs or arms trafficking. Gaol sentences for wildlife smuggling are often minimal and fines disproportionately less than the commodity values of the goods on the black market [5–8]. With little disincentive for criminal activity, the black market in wildlife continues to flourish, and places ever-increasing pressures on endangered species.

The illegal wildlife trade pose serious threats, both direct and indirect, to global biodiversity. Species sought for trade are directly impacted by over-exploitation. Over-exploitation is fuelled by the black market placing

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exaggerated values on rarer species. As a species becomes rarer from exploitation, its value on the black market escalates making it even more desirable despite the greater effort required to collect individuals from declining populations [9]. Over-exploitation of wild populations can rapidly cause local extinctions and, if harvesting is extensive across the range of the species, can cause global extinction. Widespread extinctions have occurred in taxonomic groups that are particularly vulnerable to the effects of over-exploitation because of their life history characteristics such as longevity, high natural juvenile mortality, and low reproductive outputs. For example, turtles worldwide are in peril with 3% extinct or extinct in the wild, 9% critically endangered, 18% endangered, and 21% vulnerable [10]. In Asia, the situation is even more dire with 1% extinct or extinct in the wild, 20% critically endangered, 31% endangered, and 25% vulnerable [10]. Over-exploitation of wild populations for meat, pets, and the use of the shells in traditional medicines are the major cause of declines in turtles worldwide, especially in Asia [11]. Turtles are just one of many examples of taxa that are threatened globally from over-exploitation for trade. The list of species directly threatened by wildlife trade is extensive, encompassing all major taxonomic groups across all biomes. It includes many 'keystone' species (e.g. African horn bills, sea otters, grizzly bears, sea stars, elephants, orangutans, beavers, truffles and oysters), so named because they are 'key' to the functioning of the ecosystem and their loss causes widespread declines in many other species [12–14]. Direct exploitation for hunting, trade, and collection has been identified by the World Conservation Union (IUCN) as the second greatest driver (surpassed only by habitat destruction) of declines in endangered animals, impacting 33%, 30% and 6% of threatened mammals, birds and amphibians, respectively [15]. Wildlife trade also provides avenues for the introduction of exotics with the potential to spread disease to native species [16–20] or to become invasive [21–24].

Monitoring trade in wildlife requires firstly the identification of the species traded, then assessment of whether they are derived from legal or illegal trade. Diagnostic morphological traits have traditionally been used as markers, but they are not suitable when traded products are degraded or highly processed as the morphological traits may not remain discernable. Molecular markers are ideal for species identification because unlike morphological markers they do not require intact specimens. DNA can be readily extracted from highly processed and degraded products commonly encountered in wildlife trade markets such as cooked and dried meats [25], claws left on tanned hides [26], dried shark fins [27], egg shells [28], animal hairs [29], bone [30], ivory [31, 32], rhinoceros horns [33], turtle shell [34], feathers [35] and fish scales [36].

Molecular technologies have great utility for wildlife forensics. Assigning geographic origins of trade products can also be achieved using molecular methods, a task that is often impossible using morphological traits alone. Knowledge of geographic origin can be used to distinguish between legal and illegal products, to assist in the repatriation of seized animals back to their source population, and to identify which populations are most intensively harvested for trade. At a finer resolution, individuals themselves can be marked and tracked using unique DNA profiles to characterise them. Additional information such as sex and parentage can also be ascertained which is especially useful for monitoring the compliance of registered breeders to wildlife regulations, such as to detect whether breeding stock has been supplemented or restocked with illegally caught wild stock.

In this review we detail the various contributions of genetics to wildlife forensics. The techniques employed for species identification, determination of geographic origin, individual identification and sexing will be briefly explained. Considerations for the application of these techniques to wildlife forensics will be discussed and illustrated with published case studies. To conclude, we will describe new technologies on the horizon for wildlife forensics and the future role of genetics to combat the growing global black market dealing in wildlife.

Species identification methods

Several approaches have been adopted for identification of wildlife species distinguished by the DNA target (mitochondrial or nuclear) and the technique applied to develop the genetic marker (Table 1). Some techniques, such as sequencing, can be applied to investigate both types of DNA, while other techniques are specific to nuclear DNA (nDNA).

Mitochondrial DNA (mtDNA) is often favoured as a genetic marker over nDNA for species identification of wildlife because mtDNA is easier to type from highly processed and degraded tissue. This is because mitochondria are present in multiple copies per cell compared to one copy of nDNA from each parent [37]. Method development time is typically substantially less for mtDNA markers compared to nDNA markers because universal mtDNA primers are available, which are used to amplify an informative segment of mtDNA across a wide range of taxa [38]. Amplification is done using the polymerase chain reaction (PCR, Fig. 1) [39]. Universal mtDNA markers have been successfully applied in the identification of wildlife for forensic cases. The most commonly used universal markers for species identification are the mitochondrial cytochrome b (*Cyt b*) and the cytochrome oxidase 1 (*CO1*) genes.

Table 1 Comparison of genetic markers used for forensic applications

	Species ID	Regional ID	Population ID	Individual ID	Parentage	Limitations for forensics	Advantages for forensics	Applications to generate baseline genetic data
Mitochondrial gene (mtDNA) sequencing	√√	√√	√	×	√√ maternity	Heteoplasmy	Suitable for trace and degraded DNA	Phylogenetics
					× paternity	Nuclear paralogs	Universal primers available	Phylogeography
						Maternal inheritance		Population genetics
						Single linked genome hence effectively is one single marker		
Nuclear gene (nDNA) sequencing	√√	×	×	×	×	Not suitable for trace or degraded DNA	Recommended for use in combination with mtDNA for species	Phylogenetics
						Universal primers not available for most species	Identification	
							Can detect hybrid individuals	
Pyrosequencing	√√	√ not assessed	√√ for SNP genotyping	√√ for SNP genotyping	√√ for SNP genotyping	Only short fragments of 10 to 500 bp can be sequenced	Enables very rapid high throughput genotyping of short fragments or SNPs	Population genetics for SNP genotyping
Amplified fragment length polymorphism (AFLP)	√	√	√	√	√	Dominant marker, therefore less informative for all applications	No prior genetic knowledge of the organism required	Phylogenetics, phylogeography population genetics. Limited use because of their dominance
						Not suitable for trace or degraded DNA		
Species-specific priming	√√	×	×	×	×	Knowledge of species boundaries required	Rapid screening once developed	None
							Cost effective	
Short tandem repeat (STR). Also called simple tandem repeat (SSR) or microsatellite	×	√√	√√	√√	√√	Allelic dropout can occur when trace or degraded DNA is used	Highly informative marker for many applications	Most commonly used marker for population genetics because of its high information content
						Development time is substantial	Techniques have already been validated for human forensics	
Single nucleotide polymorphism	√√	√√	√	√	√	Approx five times more loci required compared to STRs	Highly reproducible	Use of this marker for phylogenetics, phylogeography and population genetics is still in its infancy
						Currently not available for many species	Rapid screening of samples	

√√ is highly informative, √ informative, × not informative

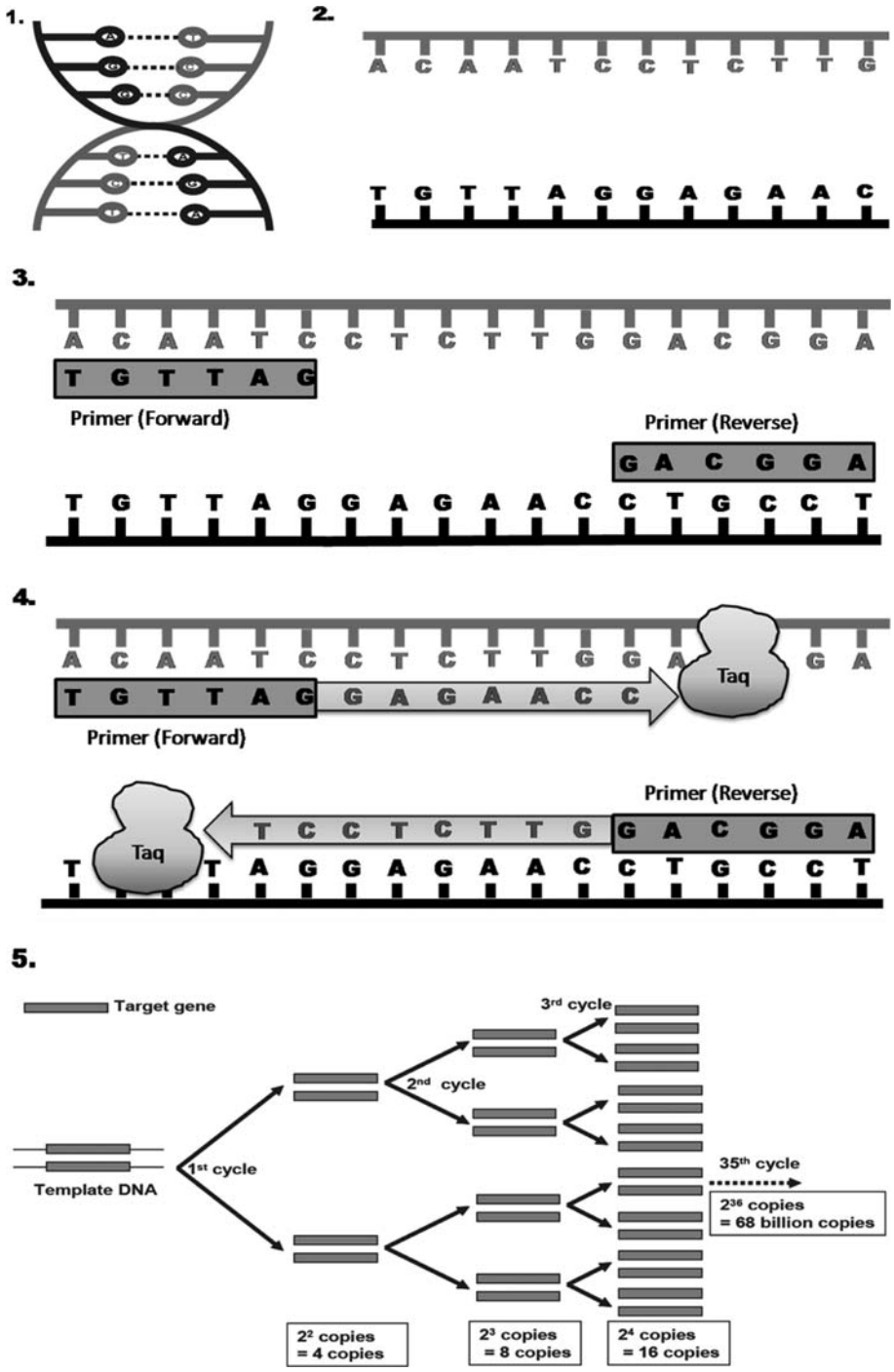

Fig. 1 The polymerase chain reaction (PCR) is a thermocycling reaction used to amplify targeted regions of DNA. Extracted genomic DNA (Step 1) is denatured by heating to 94–98°C for 20–40 s to separate the two complimentary strands of DNA (Step 2). Primers of 15–25 nucleotides in length anneal to the DNA template strand and flank the gene (or region) of interest at a temperature of 50–65°C for 20–40 s (Step 3). A DNA polymerase enzyme, such as *Taq* polymerase, with an optimal temperature of 75–80°C, recognizes the primers and synthesizes a new DNA strand complimentary to the template DNA strand. As a standard rule of thumb, at its optimum temperature, Taq polymerase can synthesize 1,000 bases per minute (Step 4). Steps 2 to 4 are repeated to produce a PCR cycle. Each PCR cycle produces an exponential increase in the number of copies of the gene (or region) of interest with approximately 68 billion copies after 35 cycles (Step 5). An entire PCR of 35 cycles is completed within 2–4 h in a thermal cycler machine

Discrimination of species using a fragment of the *Cyt b* or *CO1* genes can be based directly on DNA sequence differences between species [26, 36, 40–43] or by DNA profiling (discussed later). The *Cyt b* gene is an informative marker used in the identification of many vertebrate species from trade products including sharks [28], snakes [44], marine turtles [36], seals [45], birds [46], and tigers [30, 43, 47, 48]. Sequencing of a 600 base pair (bp) portion of the *CO1* gene has been proposed to be an efficient, fast, and inexpensive way to characterise species and an international effort is underway to use this gene to catalogue all vertebrate biodiversity on earth (www.barcodinglife.org). Pyrosequencing is an alternative method for direct sequencing of DNA templates that uses a series of enzymatic reactions to detect visible light emitted during the synthesis of DNA and enables more rapid screening of samples compared to conventional sequencing methods [48]. Only short fragments of 10–500 bp of DNA can be

sequenced with pyrosequencing methods, which can limit its application in forensics unless highly variable and informative regions are targeted [48]. Karlsson and Holmlund (2007) [49] used pyrosequencing to develop a highly sensitive assay to identify 28 species of European mammals based on short fragments of the mitochondrial 12S rRNA and 16S rRNA regions (17–18 and 15–25 bases, respectively).

While mtDNA can be effective for species identification, it does have limitations that need to be considered and overcome before it can be used for forensic application. The matrilineal mode of inheritance of mtDNA may not reflect the patterns of nuclear genetic relationships between species, particularly if there is strong sex-biased dispersal [50, 51]. Inheritance of the mtDNA genome can be complicated when paternal leakage results in heteroplasmy, that is, the coexistence of two or more different mtDNA genomes in the organism [52–56]. Nuclear paralogs (also called nuclear pseudogenes) of mtDNA genes occur when segments or the entire mtDNA genome inserts into the nucleus. These inserts can be subsequently subject to duplications, rearrangements or recombination and they may experience a different rate of mutation from that of their mtDNA parent [57–59]. Nuclear pseudogenes can be amplified simultaneously or even preferentially with the mtDNA gene in the polymerase chain reaction and the resultant mixture of genes with different modes of inheritance and mutation rate obscures any inferences of ancestry and evolutionary relationships of species [20, 59–61]. However, techniques such as sequencing of the whole mitochondrial genome can be used to test for the presence of psedogenes and once they are accounted for they may even be phylogenetically informative [60, 62]. Owing to a lack of recombination (exceptions do occur, see [54, 63]) the mtDNA genome represents a single gene history and the evolutionary history of what is effectively a single gene may not accurately reflect the species history. Multiple genes, preferably both mitochondrial and nuclear genes, are recommended for species delimitation [64–67]. Currently, markers derived from nuclear genes are not available for the majority of wildlife and consequently mtDNA approaches dominate systems for species identification. However, the advent of whole genome sequencing of non-model organisms is expected to increase the availability of nuclear genes for wildlife in the next few years. If species have been delimited then mitochondrial data can serve, and has been used, as a robust tool for species identification to provide evidence for forensic cases [30, 68–71]. Mitochondrial techniques for species identification have undergone preliminary validations studies for use in forensic application [30, 41].

DNA profiles can also be generated using the technique of PCR-restriction fragment length polymorphism (PCR-RFLP) to target specific areas of genetic variation among samples. Initially, the DNA segment of interest is amplified using PCR to generate billions of copies of the gene, and then subjected to digestion by restriction enzymes. These enzymes recognise specific base pair sequence motifs (that are often mirror images such as ATTA, GATTAG, etc.) and cut the amplified fragment at these sites. Species that differ in base composition at the restriction enzyme recognition sites will differ in whether or not the enzyme cuts the DNA. This generates DNA fragments of differing lengths (i.e. polymorphic fragments), in which the number and size of the fragments depends on the number of cutting sites in the DNA fragment of interest [72]. Electrophoresis of samples through an agarose or polyacrylamide gel separates fragments based on size and the different taxa will have characteristic banding patterns. Selection of restriction enzymes for PCR-RFLP analysis must ensure that the variability between species is appropriately represented and consequently that all species tested can be accurately discriminated from each other by their unique banding pattern, termed a RFLP profile. This technique has been successfully applied to a case in Argentina of a theft of livestock [73] and for species identification of marine turtles [29]. RFLP approaches are cheaper than direct sequencing and are suitable for forensic applications but they do not provide the baseline information that is required for the interpretation of forensic data such as the delineation of species boundaries.

For both direct sequencing and profiling approaches, phylogenetic studies (preferably with support from morphological, behavioural or physiological-based taxonomy) are necessary to form the foundation for accurate molecular species identification of wildlife. Phylogenetic studies estimate the evolutionary relationships of genes by inferring their common history and representing these relationships in the form of a phylogenetic tree. More closely related genes are in closer proximity to each other on the gene tree compared to more distant relatives, with rooting of the tree at their implied most recent common ancestor [74]. These gene trees are used to infer the phylogenetic species, although this does require careful consideration because genes can evolve in an independent manner to the evolution of the species [65]. It is also important to choose a gene with the appropriate mutation rate for phylogenetic analyses of species. A highly conserved gene will not be informative at the species level but will be better suited to the resolution of deep taxonomic relationships. Conversely, a gene that is evolving at a high rate will be informative at the level of the population or individual, but become saturated at the species level owing to homoplasy [75]. Homoplasy arises when certain nucleotide sites are subject to repeated mutation over time, and mutations back to the original state occur, or when the same mutation occurs independently in different lineages. As a result two individuals can share a derived

diagnostic base pair by chance rather than by descent [76]. When the appropriate gene region is chosen based on its rate of mutation, the levels of genetic diversity within and among species need to be sufficiently characterised before the gene can be applied to species identification of wildlife for forensic applications. This is to ensure that cryptic species—species that are morphologically indistinguishable but reproductively isolated—are represented [77]. In addition, the biological characteristics of the species also need to be considered such as their propensity to hybridize. Hybridization between species is common for many groups of taxa [78, 79]. Owing to the predominantly maternal mode of inheritance of mtDNA, these phenomena might not be reliably detected in forensic samples with mtDNA markers alone particularly if there are sex-biased hybrid compatibilities [50, 80–82]. A combination of mtDNA and nuclear markers (with their bi-parental inheritance) are recommended when hybridisation between species is suspected [80, 83–85].

Arbitrary fragments of genomic DNA (both mitochondrial and nuclear) have been used for species identification of wildlife with the major advantage that no prior genetic knowledge of the organism is required. One technique is termed Amplified Fragment Length Polymorphism (AFLP) and it generates segments of DNA of varying lengths that differ among individuals and species and can be visualised on polyacrylamide gels as series of bands. To use the AFLP technique, genomic DNA is cut at specific sites using two restriction enzymes that target common sites and produce hundreds of fragments. A subset of these is specifically selected for PCR amplification and tagged with a fluorescent dye (Fig. 2) [86]. This allows their detection by a laser by gel electrophoresis on a polyacrylamide gel. AFLP profiling has been used to discriminate between illicitly cultivated marijuana and hemp [87–90], identification of illegal hallucinogenic fungi [91–93], and for the identification of species of legally protected owls and their hybrids [94].

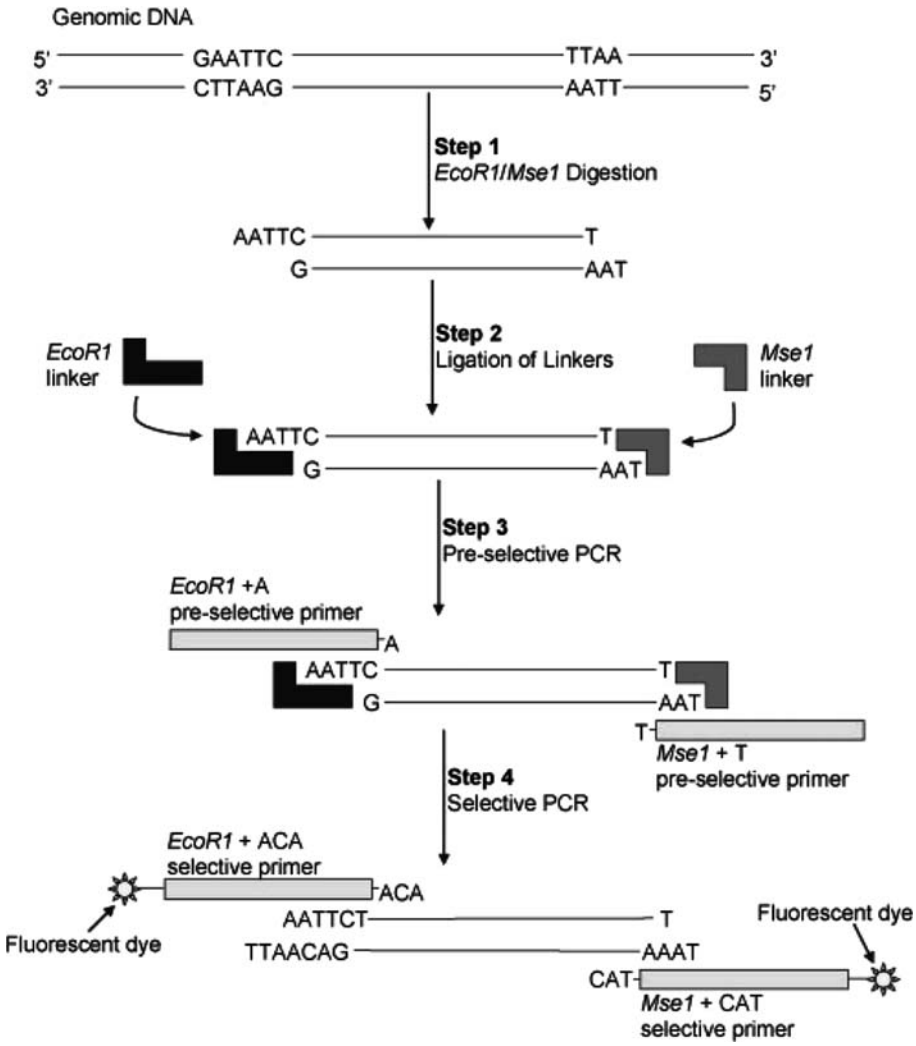

Fig. 2 The amplified fragment length polymorphism (AFLP) procedure is used to generate informative DNA fingerprints by (1) digestion of genomic DNA with a combination of two restriction enzymes, commonly *Eco*R1 and *Mse*1 for animals; (2) ligation of the double-stranded *Eco*R1- (*black*) and *Mse1*- (*grey*) specific linkers to the fragment ends; (3) a pre-selective application step using primers that anneal to the linker ends of the fragments and have one selective nucleotide at their 3′ end used to amplify *Eco*R1/*Mse*1 templates; (4) a final selective PCR in which additional nucleotides are added at the end of the *Eco*R1 and *Mse*1 selective primers; (5) Separation of fragments by migration through a polyacrylamide gel (not shown). Fluorescent dyes enable pool-plexing of AFLP reactions and are detected by a laser as the fragments migrate through the gel

Compared to mitochondrial DNA, AFLP is better suited to the detection of hybrid individuals because of its biparental mode of inheritance [95, 96]. However, AFLP is not well suited for trace samples or highly degraded samples that are commonly encountered in forensics because it requires at least 50–100 ng of high molecular weight DNA [97]. Main sources of genotyping error for AFLP are differences in the peak intensities of loci between individual runs but error rates can be minimized by genotyping replicates for 5–10% of the samples and normalizing the peak height for loci against their average intensities [98, 99]. A format for databasing and comparing AFLP profiles has been developed by Hong and Chuah (2003) in a user friendly software package which minimizes sources of genotyping error and shows great promise for use in the validation of the AFLP technique for forensic applications [99].

Sequence information from either mtDNA or nDNA can be used to develop species-specific PCR primers based on nucleotide differences between the aligned homologous sequences of the target species with other closely related taxa. These primers amplify DNA regions of the target species exclusively or have a PCR product of a characteristic size, and have been used to identify taxa of commercial interest or those commonly encountered in markets. Development of species-specific primer techniques first requires sequence data from all species likely to be encountered, for the design of putative primers. It is imperative that mixed genomic DNA samples are included in PCR tests of the development phase, to ensure that the primers are specific for the target species. Once developed, species-specific primer tests are a very rapid, sensitive and cost effective screening method to detect the presence of the target species in market products or from a mixture of genomic DNA. Species-specific primers that amplify the nuclear *ITS2* region, and the mitochondrial *Cyt b* have been used to develop assays for the identification of various shark species from dried fins or meat [28, 100–103]. Where there is no prior sequence information available for the target species, RAPD (Randomly Amplified Polymorphic DNA—where arbitary primers are used to amplify random segments of DNA) or AFLP profiling can be used to generate species-specific primers [104]. Bands specific to the target species are identified from the DNA fingerprint, extracted and sequenced. The sequence is used to design primers that will specifically amplify the species-specific region, termed a sequence characterized amplified region (SCAR). The SCAR method has the advantage of being highly reproducible: a shortcoming of the RAPD technique that has caused its redundancy in population genetic and forensic applications [105]. Genotyping individuals with species-specific primers developed using the SCAR technique is considerably cheaper than sequencing or AFLP approaches, and has been successfully used to identify deer [106], snake [44], fish [107] and fly species [108].

Regardless of how species-specific primers are developed, results that are more robust are achieved using multiplex PCR where several primers are added to a PCR to simultaneously amplify different DNA regions of the target species in a single PCR reaction. To reduce the incidence of false negatives, universal primers that amplify across all potential taxa are included in the multiplex assay. If the universal primer amplifies but the species-specific primers fails to amplify, the absence of the target species in the sample is confirmed. If both the universal and species-specific regions fail to amplify, the PCR reaction is deemed not successful and the result is inconclusive. Multiplex PCR reactions may also be used to identify several different species in a single assay. For example a multiplex PCR with six different species-specific primers and two universal shark primers for positive controls has been used to identify six species of sharks commonly encountered in North Atlantic fisheries [103]. Species-specific primers are ideal for forensic applications because they are cost effective and can be used for large scale screening of samples. However, a considerable amount of development time is required and once developed it can be difficult to incorporate additional species in the multiplex assays. Furthermore, a comprehensive understanding of species boundaries is required prior to the development of species-specific primers to ensure that all species likely to be encountered, including cryptic species, are included in the assay and can be reliably distinguished from each other.

Identification of a forensic specimen to its geographic origin

In many wildlife forensic cases, such as where commercial trade is established, the identification of the traded product or 'specimen' to the species level may not be sufficient and the geographic origin needs to be determined. Geographic origins of an individual can be identified if there is known genetic structure within the region of interest using phylogeography or population assignment methods. Phylogeographic studies assess the geographic distribution of genealogical lineages where specific mtDNA haplotypes are associated with broad geographic regions [109]. For example, phylogeographic data for four species of seahorse (*Hippocampus barbouri*, *H. spinosissimus*, *H. trimaculatus*, and *H. ingens*) were used successfully to determine the broad geographic origins of seahorses that were found for sale in traditional medicine and curio shops in California [110].

More subtle genetic differences can often be detected using population assignment methods in comparison to phylogeographic analyses. Population assignment methods are based on allelic differences at hypervariable nDNA

genetic markers between groups of individuals, also loosely called 'populations'. Assignment tests are used to estimate the probability of an individual belonging to each of these putative populations, and the forensic specimen is 'assigned' to its most probable population of origin. Conversely, exclusion tests can be used to reject the hypothesis that a specimen originated from a particular population [111–114] The hypervariable markers most often used for population assignment or exclusion are AFLPs and microsatellites (also called short tandem repeats or STRs). Microsatellites are short sequence motifs typically 1–6 nucleotides in length (e.g., ATATATAT) that have a high mutation rate predominantly due to slippage of the polymerase during DNA replication (although other mutation mechanisms have been proposed, see [115]) resulting in lengthening or shortening of the number of repeat units. Microsatellites are codominant markers in which the gene variants (or alleles) inherited from both parents are amplified in a PCR reaction and visualised on a polyacrylamide gel or CE instrument. Homozygote individuals have the same sized STR repeats (e.g., $[AT]_6$, $[AT]_6$) whereas heterozygote individuals have different sized repeats (e.g., $[AT]_3$, $[AT]_6$). In contrast AFLPs are dominant markers where an allele (or fingerprint band) is either present in the individual or absent. The heterozygosity of an individual cannot be determined directly from an AFLP band (or locus) and hence these dominant markers have far less resolving power per locus to determine the population origins of an individual in comparison to the codominant microsatellite markers [97]. Typically, at least eight microsatellite loci or 50 AFLP loci are recommended for population assignment studies [116]. The AFLP technique requires high quality DNA and hence is less versatile for degraded or trace samples in comparison to microsatellites [116]. The genotyping errors associated with AFLPs have been found to be greater than for microsatellites mainly because of differences in peak height intensity [98].

A suite of statistical analyses for assignment methods are currently used to identify the origins of individuals based on their AFLP or microsatellite profile, with the most suitable method depending on the scenario [111, 114, 116–122]. Assignment tests are highly accurate when all potential source populations have been sampled, populations boundaries are well defined, sampling is random, and populations are in Hardy–Weinberg equilibrium (i.e. there is a balance between mutation and genetic drift, no inbreeding and random mating). However, these assumptions are not realistic for many populations such as when populations are small, population boundaries are not clear or the genetic divergences between populations are low [119]. For populations with ill-defined boundaries, clustering methods perform well because they can determine the number of populations (i.e. clusters) present based on the multilocus genotypes of individuals rather than on predetermined boundaries. They then assign individuals to these identified populations, including to populations that have not been sampled [123]. Programs such as Geneland [124] can map the probabilities of an individual belonging to a 'cluster' or 'population' onto the landscape in an easily interpretable visual format ideal for the presentation of evidence to a jury in a court of law. Other methods such as spatial smoothing are most effective when the organism has a continuous distribution across the landscape and a spatial structure is not imposed (see [114] for review of assignment methods).

For example, spatial smoothing assignment has been successfully used to monitor the African elephant ivory trade by characterisation of the allele frequencies of 16 STR loci across the entire African elephants' range. Geographic-specific alleles were shown to be effective in the inference of the geographic origin of individual DNA samples with 50% identified to within 500 km of their source, and 80% to within 932 km of their source [34]. This study was later applied to a forensic case involving the largest seizure of contraband ivory since the 1989 ban on the ivory trade. A total of 532 ivory tusks, and 42,210 "hankos" which are ivory cylinders cut from the solid portion of the tusk, were found in a container shipped via South Africa to Singapore in June 2002. Assignment tests using the 16 STR loci indicated that the ivory was entirely from savannah rather than forest elephants and most likely originated from a narrow strip of southern Africa that centred on Zambia [33]. This information is invaluable for wildlife enforcement agencies to identify current poaching "hot spots" and to identify whether legally declared government stockpiles are being illegally traded and replenished [33].

Assignment tests have also been used to relocate seized animals of unknown origin back to their original population. European pond turtles (*Emys orbicularis*) are highly sought after for pets and hence are often subject to illegal collection. Specimens seized by wildlife authorities are sent to recovery centres or zoos where they rapidly accumulate in large numbers. When the turtles become too numerous to maintain in these facilities they are sacrificed or re-located to their supposed region of origin. Characterisation of three turtle populations at seven microsatellite loci assigned 22 of 36 turtles in recovery centres to their population of origin [125]. Releasing turtles that have been genotypically assigned to the population reduces the risk of outbreeding depression, which is a reduction in reproductive output and fitness that can result when two genetically distinct populations interbreed. Such targeted releases also minimize the possibility of corruption of the evolutionary processes leading to divergence among geographic isolates, an important precursor to speciation.

In a similar manner to population assignment tests, exclusion tests can be used to exclude individuals as belonging to a given population based on their allelic or genotype frequencies. An example of the use of exclusion tests to provide evidence for a wildlife related crime is the 2004 case of a suspected illegal translocation of four red deer (*Cervus elaphus*) into a hunting area in Luxembourg. Exclusion tests based on allelic frequencies for 13 microsatellite loci verified that the Luxembourg red deer were not founded from migrants from the adjacent populations of France, Belgium and Germany. Instead, they were most likely sourced from deer farms and had been illegally translocated into the area for recreational hunting [126]. Genotype exclusion tests, based on ten microsatellite markers, have also been used to successfully discriminate between hatchery-raised versus wild stocks of the commercially important marine fish red drum, *Sciaenops ocellatus*, of the south eastern United States [127].

Individual identification, sexing, and parentage

Identification of an individual based on their unique genetic profile can be used to monitor the number of animals entering commercial markets, even if they are sold as meat or highly processed products. Baker et al. (2007) [128] combined market surveys with DNA profiling to estimate the numbers of North Pacific minke whales (*Balaenoptera acutorostrata* spp.) sold in 12 markets in the Republic of (South) Korea from 1999 to 2003. A 464 bp fragment of the mtDNA control region and eight STRs were used to develop a 'DNA profile' for each market product. The DNA profiles were evaluated for matches with other profiles and the numbers of unique DNA profiles were assumed to be minimum number of individual whales sold on the market, with matching DNA profiles representing replicates from the same individual. The total number of individual whales sold over a 5 year period was estimated to be 827, almost double the officially recorded by-catch of 458 whales during this period, suggesting that illegal trade of North Pacific minke whales in South Korea is rampant [128]. Characterising individuals with unique DNA profiles is an accurate method of monitoring markets to determine what species are present, and the numbers of individuals of each species sold.

To distinguish between legally and illegally obtained specimens, a DNA register can be established where each legal specimen is DNA profiled in a certified laboratory and the profiles are lodged in a database. When there is a confiscated specimen, the DNA database can be interrogated to rapidly identify unregistered (and presumably illegally obtained) specimens. In Norway, a DNA register for minke whale has been established containing 2676 individual genetic profiles. The genetic profiles are generated using information from the mitochondrial control region, two sex determination markers and 10 microsatellite loci. The Norwegian minke whale DNA register has proven to be effective in verifying legal specimens by consistently matching 20 specimens of minke whales obtained from Norwegian markets to reference samples in the register [129]. An effective DNA register requires all legal specimens to be lodged and genotyped using highly sensitive hypervariable markers, such as microsatellites, that have the resolution to differentiate between individuals. Wildlife DNA registers can also be used to monitor the compliance of breeders to ensure that captive bred stock are not being replenished or supplemented from illegally caught wild stock. The offspring of captive breeding stock can also be verified by assessing the parentage using a suite of hypervariable microsatellite markers, similar to methods used for human paternity analyses [69].

Determining the sex of an animal can be difficult for some taxa where differences between the sexes are not obvious or the illegally killed carcass is decomposed. Determining the gender of the Asian elephant is important because tusks are only present in males and drastic declines in the numbers of males from hunting for their ivory can result in unbalanced sex ratios in the population. It can be difficult or impossible to determine the sex of Asian elephants when the carcass is decomposed, but a simple and inexpensive test based on the SRY gene on the Y chromosome has been developed specifically for identifying male Asian elephants from poached carcasses [130].

In some countries, qualified hunters are restricted to hunting only one sex and monitoring the trade requires determination of the sex of the animals hunted. In Korea the hunting of female pheasant is illegal and sex-specific markers have been used to identify illegal hunting. In one case (February 2004), five pheasant carcasses were found in a suspect's refrigerator. Using two avian sex-specific markers, one marker on the Z chromosome and one on the W chromosome, gender could be determined because avian males are homozygotes (ZZ), whereas females are heterozygotes (ZW). Two of the five pheasant carcasses were female and the suspect was subsequently prosecuted for illegal hunting based on the DNA evidence [68].

Method validation

Genetic techniques need to be validated for use in forensic applications. This is not a trivial matter either at a scientific level or legal level. The use of DNA markers for wildlife forensic application need to be tested against what has become the 'gold standard' for forensic science, the validation of human DNA. Butler (2005) defines validation as

the process of demonstrating that a laboratory procedure is robust, reliable, and reproducible in the hands of the personnel performing the test [131]. A robust method is one in which successful results are obtained in a high percentage of tests at first testing. A reliable method is one in which the results are accurate and correctly reflect the sample being tested, and a reproducible method is one in which the same result is obtained each time a sample is tested. All three properties are important for techniques performed in forensic laboratories [131]. To meet these exacting standards, forensic scientists need to document (a) full details of the tests used to validate new techniques; (b) the technical procedures and policies to instil confidence in the laboratory processes and policies; and (c) the policies relating to the interpretation of data.

In the forensic world, most DNA analysis is conducted using commercially available technologies, reagents and 'kits'. The *development validation* of the latter will have been carried out by the commercial entity prior to product release. Hence, the forensic laboratory is required to carry out more limited *internal validation* (also called verification) aimed at showing the laboratory can meet accepted validation requirements [131].

Development validation is an exacting process and several organisations at an international level have defined the standards for forensic application. The most commonly used standards are these developed by SWGDAM (Scientific Working group on DNA Analysis Methods) and the ISO17025 or IEC17025 laboratory accreditation. This group and laboratory accreditation was first established in the late 1980s under FBI sponsorship to aid forensic scientists as DNA applications in forensics first emerged. A detailed discussion of these and similar guidelines is beyond the scope of this paper, but they include testing the technology for consistency and reproducibility against standard samples, samples in more complex matrices, mixed samples and samples exposed to a variety of environmental conditions. These criteria are aimed at ensuring the technologies are robust in producing reliable results with real life forensic samples. SWGDAM also makes recommendations with regards to population studies and data interpretation [132].

It can readily be appreciated that forensic validation studies are onerous and time consuming. There have been few comprehensive attempts to validate non human DNA tests for forensic applications. One recent major study validated DNA markers for Cannabis plant samples [133] and this work gives a useful insight to the challenges which will face scientists seeking to cross the bridge between research and development to professional application in forensics.

The admissibility of evidence in the legal system is governed by different rules which also need to be met. These vary according to the legal frameworks and systems in different countries. In much of the United States, and also followed by many other countries, scientific evidence must meet the Daubert standards [134] under which scientific techniques must have:

- been tested before;
- been subject to peer review and publication;
- standards which can verify the reliability of the technique;
- known potential error notes; and
- gained widespread acceptance in the scientific community.

Scientists seeking to bring wildlife forensics into the court system need to be aware of the legal framework and rules, and the role and expectations for the expert witness within their jurisdiction. These are not trivial matters.

Future directions of genetic markers in wildlife forensics

The advent of whole genome sequencing of non-model organisms will greatly increase the markers that are currently available for forensic genetics of wildlife. Universal nuclear primers that can amplify informative regions over a broad range of taxa will become more readily available, and will complement current initiatives such as the mtDNA barcoding of life project (www.barcodinglife.org). In addition, the availability of single nucleotide polymorphisms (SNPs) that are informative for species identification, population assignment, and individual identification of wildlife will increase considerably. SNP techniques target multiple regions of the genome where single base pair mutations have occurred and they have the major advantage compared to other forensic genetic methods of being easily amplifiable from highly degraded material and are highly reproducible across different laboratories [135–138]. Furthermore, SNPs are amenable to multiplexing of up to 50 loci on a microarray platform enabling rapid and high throughput screening of forensic samples [139]. SNPs have already proven to be effective for forensic identification of the population of origin for Chinook salmon [140] and show great promise as a genetic marker to contribute to existing forensic genetic technologies.

Whilst emerging technologies will add to the forensic genetic toolbox, current genetic technologies are capable of addressing most forensic questions as evidenced by the suite of methods discussed in this review which have been successfully applied to wildlife forensic cases. The choice of genetic marker will depend on the forensic question to be addressed and the ecology, biology and genetic knowledge of the species. Each genetic technique has it

advantages and limitations for forensic applications and these must be carefully evaluated when choosing a marker (Table 1). To overcome the limitations of the genetic techniques the most powerful approach is to use a combination of complimentary markers with the appropriate resolution to address the forensic outcomes.

Ecological, biological, and genetic knowledge of wildlife has traditionally been covered in the disciplines of wildlife ecology, physiology and conservation genetics and it is this research that forms the foundation for the interpretation of genetic data for forensic applications. We argue that for forensic science to advance in the field of wildlife biology, cross-disciplinary collaborations with ecologists, biologists and conservation geneticists are essential.

Phylogenetic, phylogeographic and population genetic studies are required for species, population and individual identification of wildlife, respectively. The objectives of conservation research are often complimentary with forensic outcomes. For example, phylogenetics can be used to delineate species boundaries and this is important for the enforcement of wildlife legislation, which recognises and protects groups that are classed as 'species' or 'subspecies'. In addition, the markers developed for phylogenetics can also be used, or modified for use, for forensic species identification (Table 1). Phylogeography and population genetic studies not only provide baseline data that is required for assignment of individuals to their geographic source of origin, but are also used to identify populations that are most vulnerable to extinction from overexploitation. The markers used for these same studies can be applied to elucidate the source of traded specimens and thereby identify "hotspots" for illegal collection where enforcement efforts can be directed.

Forensic scientists can greatly benefit from liaison with conservation geneticists to collaboratively develop genetic technologies that will benefit the conservation and management of traded species, and to extend these technologies for use in a forensic context to monitor trade activities and provide DNA evidence that can be presented in court for cases of illicit trade of wildlife. Cross-disciplinary collaboration in the initial planning phase of the research programmes will foster the development of new technologies that have greater versatility with applications for both conservation and forensics.

Key points

1. The illegal trade of wildlife is worth more than USD $20 billion per year and poses a major threat to biodiversity.
2. DNA technologies are well suited to detect and provide evidence for cases of illegal wildlife trade because they can identify the species, geographic origin, individual identity, parentage, and sex of the confiscated specimen.
3. Current DNA technologies for wildlife are capable of addressing most forensic questions but further research is required to validate their routine use for forensic application and admissibility as evidence.
4. Studies of phylogenetics, phylogeography, and population genetics are complementary, and in many cases required, for the development of DNA-based forensic identification tools for wildlife.

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