Animal Forensics and Applications

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Abstract

Forensic science is the application of a broad spectrum of sciences and technologies to investigate situations after getting the facts and to establish what occurred based on collected evidence. This is especially important in law enforcement where forensics is done in relation to criminal or civil law. In civil actions, forensics can help resolve a broad spectrum of legal issues through the identification, analysis, and evaluation of physical evidence. The field of forensic science covers document examination, DNA analysis using electronic or digital media, fingerprinting, autopsy techniques, forensic engineering, forensic anthropology, pathology, economics, accounting, biology, entomology, toxicology, and much more. In this chapter we have described different materials such as hair, blood, bone, teeth, saliva, nails, feathers, skin, leather, sperm, feces, and urine and different methods for extracting DNA from different sources. The applications of animal forensics can be broadly viewed in the following four categories such as animals can be the victim, can be the perpetrator, and can be the witness and wildlife forensics. Molecular animal forensics provides different genetic tools such as DNA sequencing, single nucleotide polymorphism (SNP), PCR-RFLP, and microsatellite analysis for species identification and for characterization or identification of a sample recovered from a crime scene or illegal wildlife traders and black markets involved in wildlife trade. The genetic identification can be done as species identification, identification of geographic origin, individual identification, etc. Mitochondrial and nuclear markers can be used for

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genetic identification of the species. Identification of geographic origin is done by phylogeography and population assignment methods. To summarize, various techniques of individual identification, sexing, and parentage can be used. These techniques involve the microsatellite genotyping, DNA nucleotide sequencing, SNP typing, RAPD, and AFLP.

20.1 Introduction

Forensic science is the application of a broad spectrum of sciences and technologies to investigate and establish facts of interest about criminal or civil law. It is a specialism that aims to help judges solve legal issues, not only in criminal law but also in civil cases. It is a very broad field, crossing the boundaries between biology, physics, chemistry, and mathematics and including disciplines as varied as ballistics and botany. It also includes disciplines like veterinary sciences, genetics, pathology, and morphology. Forensic science finds applications in both humans and animals. In case of humans, it is more often used in human identity testing which has applications in crime solving, paternity testing, identifying accident victims and soldiers in war, solving inheritance claims, etc.

Animal forensics can be described as scientific tests or techniques used in the investigation of crimes against animals (domestic animals, agricultural animals, wildlife, etc.). In case of animals forensic science has much more varied applications. It helps in solving cases related to crime against animals, like animal poaching and blood sport. It also helps in preventing illegal trafficking of regulated species in the form of parts and products; settling legal claims related to animals; investigating unknown causes of death to endangered, threatened, and protected animal species; etc. Since animals also form a part of human diet, and there are many religious issues concerned with the diet, animal forensics thus help in protecting social and religious values of a society. Determining the species origin of animal tissues such as in cases of illegal commercialization and poaching of game animals is also one of the applications of animal forensics.

Conventional methods used in animal forensics include microscopic examination, protein-based assays, isoelectric focusing, and enzyme-linked immunoassays (ELISA). Before 1975, the application of forensic science protocols to animal and wildlife-related evidence was mostly limited to tentative family, genus, and species identifications of blood stains and loose hairs found at human crime scenes. These methods were usually based upon immunosorbent assays tests using relatively nonspecific antisera and microscopic comparisons. However, the results were not much useful in trying to link suspect, victim, and crime scene. Furthermore, in order to enforce the CITES (Convention on International Trade in Endangered Species Fauna and Flora) (www.cites.org) regulations in courts of law, the need for species-specific identifications was felt to address an underlying forensic issue: that illegal trafficking of regulated species would be in the form of parts and products. Thus, the need for wildlife forensics on an international scale was born.

There are other challenges to animal forensics as well. These include scant availability of samples (pieces of bones, flesh etc.), very old and degraded samples, samples obtained from closely related species, etc. Over the past 20 years, however, one particular biological tool has managed to address all these concerns and, in fact, revolutionized forensic investigations—the analysis of DNA. Since all living things contain DNA, and all DNA exhibit variability both among and within species, any biological material associated with a legal case carries in it information about its source. DNA analysis has evolved to become an indispensable tool of modern forensic science including animal forensics, employing extremely sensitive PCR-based techniques to analyze biological materials. Suspects can be linked to crime scenes using DNA evidence from as little as the saliva on a cigarette butt or skin cells on a steering wheel. Similarly, cases can now be solved decades

after investigations were begun by analyzing degraded DNA from stored swabs or by analyzing DNA from degraded samples.

DNA-based forensic methods are much more efficient than the conventional methods because of so many obvious reasons. DNA is a stable molecule and contains identical genetic information irrespective of the origin of sample as far as the sample is taken from a same individual. The information content in DNA is species and/or individual specific and is more in DNA molecule as compared to that in protein molecules. Moreover, DNA-based forensic methods make it possible to deduce phylogenetic/evolutionary relations. In tropical countries, like India, due to high ambient temperature sample degradation occurs quickly. Moreover, particularly in India, there are fewer diagnostic laboratories, and so the samples have to be transported over considerably larger distances before reaching the laboratories. Also the field personnel are inadequately trained for collection, preservation, and transport of samples from collection sites to the laboratories. All these factors highlight the importance of DNA-based forensic methods.

This chapter mainly focuses on animal forensics based on DNA analysis and is divided into the following sections: materials used in forensics and protocols for extracting DNA from such materials, applications of animal forensics, and forensic genetic identification methods.

20.2 Material Used in Forensics

20.2.1 Hair

Comparative morphology, microscopy, and histology represent the classical methods in the field of forensic hair analysis. Type, number, and state of preservation of seized hair effect its value as trace evidence. Each mammalian species has hair with characteristic length, color, and root structure and various morphological characteristics. A typical hair consists of a root and hair shaft, which is basically composed of mark, cortex, and cuticle. The structure of the mark and of the hair cuticle is strictly species specific. The structure of the mark cells, the thickness of the marks and its continuity, cuticular pattern, medulla type, and medullary index also allow species differentiation. Microscopic analysis of hair roots allows not only the determination of growth phase but also a distinction between pulled out and naturally shed hairs.

20.2.1.1 DNA Extraction from Human Hairs

Hairs contain extremely small quantities of DNA (Higuchi et al. 1988). Since the content of nuclear DNA is too small for amplification, particularly those from naturally shed hairs or hair shafts rather than hair root, many studies have employed relatively abundant mtDNA (Sullivan et al. 1992; Baker et al. 2001; Pfeiffer et al. 1999; Vigilant 1999). Moreover, DNA extracted from hair is not always successfully amplified by PCR, suggesting the presence of PCR inhibitors in the extracted samples. It has already been revealed that the hair pigment melanin is a strong inhibitor of the PCR process (Yoshii et al. 1992, 1993; Wilson et al. 1995). More specifically, hair-dyeing was found to have a strong influence on PCR. In a study three different methods of DNA extraction were evaluated, and Chelex method was recommended for PCR experiments in view of its simplicity and cost-effectiveness (Suenaga and Nakamura 2005). The protocol for genomic DNA extraction recommended by them is described below.

Take two hair root segments (1 cm in length) and wash with 500 μl of 100 % ethanol in a small polypropylene test tube. After air-drying, place the hair in a 1.5-ml micro-centrifuge tube. Add 200 μl of 5 % Chelex® 100 and 10 μl of 10 mg/ml Proteinase K to the two pieces of hair placed in a 1.5-ml micro-centrifuge tube and mix well. The solution is incubated at 55 °C for at least 6–8 h or, alternatively, overnight. The mixture is vortexed and incubated in a boiling-water bath for 8 min. After centrifugation at 10,000–15,000 × g for 2–3 min, the supernatant is transferred to another 1.5-ml micro-centrifuge tube and is used for PCR amplification.

Chelex method is eco-friendly, cost effective, simpler, quick, and easy to perform and can also be used for DNA extraction from blood (Fig. 20.1), skin, feather, bone, muscle tissue, fecal sample, etc.

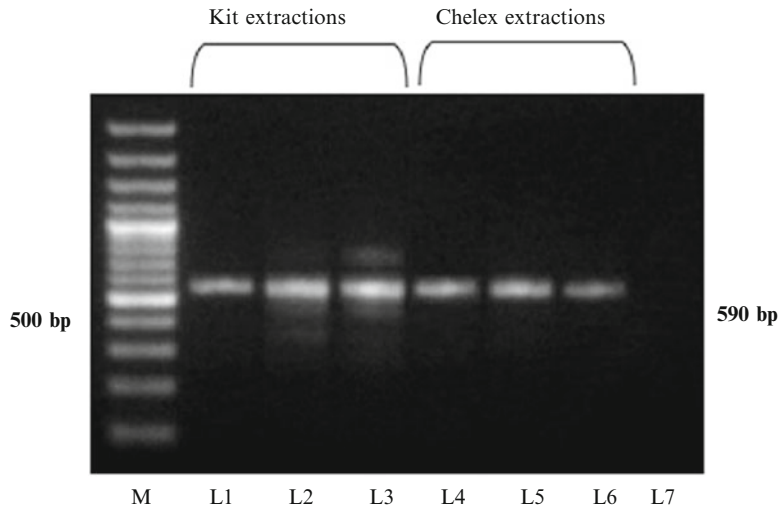

Fig. 20.1 PCR-amplified product of tiger blood DNA extractions (using kit and Chelex method) in 1.5 % agarose *M*: 100-bp ladder (MBI Fermentas); *Lanes 2–4*: Kit genomic DNA extractions; *Lanes 5–7*: Chelex genomic DNA extractions

20.2.1.2 Mitochondrial DNA (mtDNA) Extraction from Compromised Human Hairs

Since mtDNA is present in hundreds to thousands of copies per cell, it may therefore be better suited than nuclear DNA for ascertaining genetic information in cases where DNA amount may be limited, degraded, or both. The most polymorphic region of the human mtDNA genome is concentrated in two hypervariable segments within the noncoding, D-loop region (Greenberg et al. 1983). Analysis of DNA from ancient and historical anthropological samples has employed a silica-based method for DNA extraction (Hoss and Paabo 1993). In a study successful application of silica-based DNA extraction to compromised (water decomposed, incinerated, putrified) forensic hair shaft was carried out (Baker et al. 2001). The method described by them is given below.

Take 2 cm of a single strand of hair (cut hair shafts without the hair root), and wash with detergent in an ultrasonic water bath (Wilson et al. 1995). Place it in a pretreated 0.2 ml glass tissue homogenizer (Kontes Glass, Vineland, NJ) and grind in 100 µl of extraction buffer [10 M guanidine thiocyanate (GuSCN), 0.1 M Tris–HCl (pH 6.4), 0.2 M EDTA (pH 8.0), 1.3 % Triton X-100]. Transfer the homogenate to a UV-irradiated micro-centrifuge tube and pool with another 100 µl of extraction buffer used to rinse the homogenizer. Incubate the samples at 60 °C with slight agitation overnight (10–24 h). Isolate the DNA using the GeneClean II kit (Bio 101, Vista, CA). Add three volumes of sodium iodide and 5 µl of GlassMilk® to the sample, and incubate at 57 °C for 15 min with slight agitation. Following incubation, centrifuge the sample for 5 min at 12,000 × g and wash twice with the Bio 101 New Wash® solution. Remove any remaining New Wash® by centrifugation for an additional 3 min. Elute the sample in 30 µl of 10 mM Tris/1 mM EDTA buffer (pH 7.6) (TE) and incubate at 56 °C for 10 min. Centrifuge the sample again for 5 min and remove the supernatant and store at −20 °C until amplification.

20.2.2 Blood

The classical analytical methods of blood include blood group serology, the determination of serum proteins and isoenzymes, as well as the

characterization of MHC antigens. However, current analysis possibilities of blood include the whole spectrum of molecular methods. The DNA is extracted from the nucleated white blood cells. The choice of the appropriate method of analysis depends on the quantity and quality of the available sample.

20.2.2.1 Extraction of Genomic DNA from Dried Blood Samples

Due to its ease and convenience in collection and transportation, even from geographically isolated populations, dried blood samples have become very popular for forensic as well as other public health purposes. It has been shown that any biological markers that can be measured from whole blood, serum, or plasma can be determined from dried blood specimens (Mei et al. 2001). In a study, Nguyen et al. (2012) developed a two-step lysis method for genomic DNA extraction from dried blood samples. The method was developed based on the Sumota Chaisomchit one-step lysis method (Chaisomchit et al. 2003). The two-step lysis method was shown to be more efficient and cost effective as compared to the one-step lysis method and commercial QIAamp® DNA Mini Kit-based method. The protocol for the two-step lysis method is described below.

Punch out three pieces of 3-mm-diameter circle from the dried blood spot samples into a 1.5-ml micro-centrifuge tube. Vortex with 200 µl of lysis buffer I (Tris–HCl 10 mM, MgCl2 5 mM, Triton X100 (1 %v/v), SDS 1 % w/v, EDTA 10 mM, and adjusted with pH 8.0) for 30 s and then incubate at 85 °C for 20 min. Cool down the lysate at room temperature for 10 min. Add 0.01 mg of Proteinase K and vortex again for 30 s. Add 100 µl of lysis buffer II (Tris–HCl 30 mM, EDTA 20 mM, SDS 3 %, and adjusted with pH 8.0). Vortex again for 30 s. Incubate at 65 °C for 1 h. Add the same amount of buffer phenol:chloroform:isoamyl alcohol (25:24:1) and mix well for 30 s. Centrifuge at 10,000 rpm for 4 min at room temperature and transfer the upper phase into a fresh tube. Treat with sodium acetate (3 M, pH 5.2) and isopropanol. Mix and centrifuge at 10,000 rpm for 4 min at room temperature. Remove supernatant and wash the pellet with 70 % ethanol. For long-term use, the pellet can be stored in 70 % ethanol at −20 °C. Prior to use, however, the pellet is centrifuged to remove the washing and dried at room temperature until there is no trace of ethanol. The genomic DNA is then resuspended in 50 µl of TE buffer [10 mM Tris, 1 mM EDTA].

20.2.3 Saliva

Fresh whole blood or blood-stained material is the primary source of an individual's DNA, however, in a study, Walsh et al. (1992) presented several methods for isolating DNA from saliva and saliva stains and showed that saliva can serve as an alternative source of DNA for known standards. They isolated DNA from fresh saliva and various saliva-stained materials (stored under different conditions), such as envelopes, buccal swabs, gags, and cigarettes (Walsh et al. 1992). The various methods described by them are described below.

20.2.3.1 DNA Extraction from Saliva

Take fresh saliva and centrifuge for 1 min. Resuspend the pellet in 0.7 ml of lysis buffer [10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8.0), 10 mM ethylenediaminetetraacetate (EDTA), 0.1 M sodium chloride (NaCl), and 2.0 % sodium dodecyl sulfate (SDS)] (Gill et al. 1985), and add 35 µl of 20 mg/ml Proteinase K. The saliva cell pellets can also be resuspended in 305 µl of 10 mM Tris (pH 7.6), 10 mM NaCl, 1 mM EDTA, 1 % SDS, and 0.65 µg/ml Proteinase K in the presence or absence of 39 mM dithiothreitol (DTT). If saliva is frozen at −20 °C, thaw it at 20 °C and use 1 ml aliquot for pellet formation and then treat with lysis buffer as above.

In case of buccal swabbing, remove cotton from the swab stem, and transfer to a micro-centrifuge tube. Add 0.7 ml of lysis buffer and 35 µl of Proteinase K (20 mg/ml). To obtain DNA from the cigarette butts, remove the filter ends of the cigarettes, cut into small pieces, and incubate in lysis buffer with Proteinase K (1 mg/ml final concentration) in a micro-centrifuge tube as above. Similarly, in case of stamps and the gummed edges of envelopes, cut into small pieces, and then transfer to micro-centrifuge tubes or 15 ml

polypropylene tubes. Add lysis buffer to cover the samples, and make Proteinase K to a final concentration of 1 mg/ml.

The rest of the protocol is the same for all the above sample types. Incubate the samples overnight at 56 °C. Extract DNA with an equal volume of phenol/chloroform (1:1) and with an equal volume of chloroform. Add 1 µl of glycogen (20 mg/ml), and precipitate the DNA with an equal volume of isopropanol at −20 °C overnight. Pellet the DNA, wash with 80 % ethanol, air-dry, and resuspend in restriction buffer [60 mM Tris (pH 7.5), 10 mM magnesium chloride (MgCl), 100 mM NaC1, 35 mM 2-mercaptoethanol, and 1 mg/ml bovine serum albumin].

20.2.4 Bone and Teeth

The protein-mineral matrix of bone poses an effective physical and chemical barrier to environmental deterioration and biological attack. Therefore, bone and teeth samples are often the only, and almost always the best, biological material available for DNA typing.

20.2.4.1 DNA Extraction from Blood and Teeth

In a study, Loreille et al. (2007) presented a highly efficient protocol for the recovery of DNA from bone and teeth by full demineralization, resulting in full physical dissolution of the bone powder and quantitative recovery of all DNA released by the complete demineralization procedure (Loreille et al. 2007). They showed that the protocol significantly enhances the quantity of DNA that can be extracted and amplified from degraded skeletal remains. The protocol is described below.

Using an aluminum oxide sanding stone, extensively sand the entire surface of each bone or tooth sample to remove potential exogenous DNA. Sonicate the samples in 20 % bleach for 5 min. Following the bleach wash, rinse the bones in UV-irradiated water, and then sonicate again for 5 additional minutes in UV-irradiated water. Perform a final sonication wash step again using 100 % ethanol, and place the bone in a sterilized fume hood to air-dry overnight.

The next day, powder the samples in a sterilized process. Take 0.6–1.21 g of finely ground bone powder, and incubate overnight in 9–18 ml of extraction buffer (EDTA 0.5 M, 1 % lauryl sarcosinate) and 200 ml of 20 mg/ml Proteinase K, in a rotary shaker at 56 °C. Extract DNA with an equivalent volume of phenol:chloroform:isoamyl alcohol (25:24:1). Concentrate the supernatant to a volume slightly less than 2 ml using centrifugal filter units (e.g., by using Centriplus from Millipore). Transfer the remaining 2 ml of DNA extract into a Centricon 30 centrifugal filter unit (Millipore) and wash three times with irradiated water (UltraPure™ DNase/RNase-Free Distilled Water, Invitrogen).

This extraction protocol includes complete demineralization of the bone/tooth powder and significantly increases DNA yields, therefore, improving DNA typing results from degraded skeletal elements.

20.2.5 Nails

Nail clippings can be used as an alternative source of genomic DNA. They are often superior to other biological specimens because nail clippings offer long-term stability, even at room temperature, and are easily transportable. Moreover, as a biological specimen, nail clippings have low infectivity and can easily be obtained from subjects of any age, at any time, and in any place.

20.2.5.1 DNA Extraction from Nails

In a study Cline et al. (2003) developed a simple and efficient method for discrete isolation and purification of nail DNA and DNA from exogenous sources (exogenous material potentially originating from an attacker during self-defense) (Cline et al. 2003). The protocol is described below.

20.2.5.1.1 Exogenous DNA Isolation

1. Soak nail in 200 µl sterile 25 mM EDTA (in water) at room temperature for 1 h. Gently vortex periodically. Transfer liquid to a new tube.

2a. To this solution add 20 µl 10 % SDS and 1 µl 20 mg/ml Proteinase K. Incubate at 50–60 °C overnight. Extract DNA using phenol/chloroform as described in step 6 below.

20.2.5.1.2 Nail Preparation and DNA Isolation

2b. To the nail add 200 µl 1 % SDS/25 mM EDTA and 1 µl 20 mg/ml Proteinase K. Vortex and incubate for 1 h at room temperature.

3. Pipet or pour off liquid, and rinse nail 5–10 times with high-quality (18.3 M-ohm) sterile water. Following a final rinse with sterile water, centrifuge nail briefly and pipet off all remaining liquid.

4. Add 200 µl 2N NaOH to nail. Incubate overnight at room temperature. Vortex periodically if desired.

5. Following incubation, vortex nail to ensure it is completely solubilized. Neutralize solution (to pH 6–8, checked by spotting 1 µl onto pH paper), by adding 100 µl of 200 mM Tris (pH 7–8) and 34.5 µl concentrated HCl. Vortex immediately. If the pH is too low a precipitate will form. Adjust pH with dilute NaOH as needed to redissolve precipitate. (Note: the 34.5 µl of concentrated HCl (11.6N) is equal-normal with the 200 µl of 2N NaOH. If the HCl is more dilute, the volume added should be increased accordingly. Old HCl may work poorly.) Continue to step 6 or 7.

20.2.5.1.3 Organic Extraction

6a. Add an equal volume of phenol/chloroform (or PCI) to neutralized sample, vortex, spin at high speed for 5 min in a micro-centrifuge, and transfer aqueous (top) layer to a clean tube. Repeat this extraction if the aqueous layer is not clear.

6b. Precipitate DNA by adding 1/10 volume 3 M sodium acetate and 2 volumes of 95 % ethanol. Incubate at −20 °C for 1 h or longer.

6c. Centrifuge at high speed for 15–30 min. Note location of DNA pellet, which may or may not be visible. Carefully pipet off all liquid. Dry pellet and resuspend in TE (10 mM Tris/1 mM EDTA) at 10 µl/mg nail.

20.2.5.1.4 Microcon-100 Purification

7a. Add an equal volume of TE to the neutralized sample and place on column. Centrifuge at 500 × g for approximately 20 min or until liquid is pushed through. Discard flow-through.

7b. Add 200 µl TE to top portion of column and centrifuge at 500 × g as in step 7a. Repeat step 7b once.

7c. Collect retentate containing DNA (generally 10–20 µl remaining on top of column; add TE if needed to 10 µl/mg nail).

20.2.5.1.5 Removal of Nail Polish

Following step 1 above, add 100 µl acetone to nail, vortex, and soak at room temperature for 10 min. Draw off acetone, discard, and repeat. Allow nail to dry and continue to step 2.

20.2.6 Feathers

The use of feathers simplifies the sampling of avian genomic DNA, especially when blood extraction is difficult because of the age or the size of the bird. It also minimizes the stress on the bird. Moreover, it is a noninvasive method and is useful particularly in case of juvenile birds and small parrots in which blood extraction is very difficult because of the small size of the blood vessels.

20.2.6.1 DNA Extraction from Feathers

In a study Volo et al. (2008) presented an improved and modified protocol for extracting DNA from feathers (Volo et al. 2008). They used the protocol to successfully isolate DNA from molted feathers. The protocol is described below.

20.2.6.1.1 Feather Preparation

Wipe all surfaces with 10 % bleach. Run UV lights (if available) for 20 min. Heat water bath to 55 °C. Use of nitrile gloves helps to reduce static electricity that causes difficulty in handling feather material. Separate calamus tip from the rest of the feather and place in a tube. Make sure to cut above the superior umbilicus, so you can include it later. Fill tube with 70 % ethanol, and soak for 30 min. Set out more 1.5 ml centrifuge tubes and fill with double-distilled water; soak feathers for 30 min. Label 1.5 ml centrifuge tubes which will now serve as digestion tubes.

Use sterile petri dish as a catching surface and sterile scissors and sterile forceps for each sample. Cut feather rachis longitudinally along its length. Stop before superior umbilicus. Then cut horizontally ~5 cm of the feather tip into 2–3 mm pieces and place into tube. Cut out the superior umbilicus and place in tube. Cut it out as a small square around the "blood-dot." Pull out the papery material from inside the calamus and place in tube.

20.2.6.1.2 DNA Extraction

Prepare the following reagents:

1X TNE: Mix 100 mM NaCl, 50 mM Tris, and 25 mM EDTA. Bring solution to pH 7.5 with HCl, and then autoclave.

Digestion mix: To prepare 795 µl (volume required for each extraction) of digestion mix, mix 600 µl 1X TNE, 60 µl 1 M Tris–HCl, 45 µl Proteinase K (20 mg/ml), 10 µl of 25 % SDS (weight/volume), and 80 µl 1 M DTT (newly mixed).

Pipet 700 µl digestion mix to each tube. Incubate at 55 °C until most of the material dissolves, overnight to 1 week, usually 3–4 days. If material is not completely digested in 2 days, add more Proteinase K (20 µl of 25 mg/µl). Cool to room temperature after digestion is complete. Pipet 233 µl protein precipitation mix (7.5 M ammonium acetate) to each tube. Pulse-vortex to mix. Place in a freezer (−20 °C) for 30 min. Centrifuge (4 °C) at 13,000–16,000 rpm for 30 min. If some debris is still floating, recentrifuge for 10 min. Pour supernatant into a new 1.5 ml tube already containing 600 µl 100 % isopropanol (DNA grade). Add 1 µl glycogen (DNA carrier) to each tube. Mix samples by inverting tubes 50 times. Place in freezer (−20 °C) overnight. Centrifuge (4 °C) at 13,000–16,000 rpm for 30 min. Look for pellet at bottom of tube. Pour off supernatant and drain tube on clean, absorbent paper. Add 600 µl 70 % ethanol (DNA grade). Centrifuge (4 °C) at 13,000–16,000 rpm for 2 min. Carefully pour off ethanol. Air-dry tubes to remove all excess ethanol. It may be necessary to leave overnight to dry. When dried add 20–50 µl TE (10 mM Tris, pH 8.0, and 1 M EDTA pH 8.0) to rehydrate pellet, flicking tube to mix. Let it rehydrate overnight before use, or warm (35 °C) in a heating block to facilitate resuspension. Use 1–2 µl for PCR.

20.2.7 Skin and Leather

Once tanned it becomes extremely difficult to differentiate skin/leather of different species or skin of a wild animal from that of a domestic animal. Taking advantage of this fact, commercial products obtained from wild animals are sometimes sold as if they were from domestic animals and vice versa. In such cases, DNA analysis is the only solution for differentiating between processed skin samples of different species.

20.2.7.1 DNA Extraction from Skin and Leather

In a study, Ojeda et al. (2012) introduced a novel DNA extraction protocol for leather samples (Ojeda et al. 2012). DNA was successfully isolated from hides in various states of preservation such as raw hide and dry salted hide and skin. The protocol which is a modification of Sambrook et al. (1989) is described below.

Place a small tissue sample of 25 mg in a 1.5-ml tube. Add 500 µl lysis buffer (50 mM Tris–HCl, pH 8.0, 50 mM EDTA, 1 % SDS, and 50 mM NaCl) and 5 µl Proteinase K (20.1 mg/µl). Incubate at 55 °C in a water bath for 8 h with stirring. Centrifuge the samples for 20 min at 13,000 rpm. Remove 500 µl supernatant (avoiding the pellet as well as the superficial oily layer if any), and place in a new tube. Add 300 µl 5 M NaCl, and centrifuge the samples for 15 min at 13,000 rpm. Finally, recover 500 µl of the supernatant, and precipitate DNA by the addition of an equal volume of absolute isopropanol. Shake samples briefly, and then vortex thoroughly. Centrifuge tubes at 13,000 rpm for 15 min, and discard the liquid with care not to disturb the pellet. Wash pellet with 750 µl 70 % ethanol, and centrifuge for 5 min at 13,000 rpm to remove the alcohol. Dissolve DNA in 25 µl 1X TE buffer.

20.2.8 Sperm, Feces, and Urine

In contrast to human forensics, the molecular analysis of semen of animals plays hardly any role. The investigation of urine is useful in doping cases. The examination of feces has practical role in tracking wildlife.

20.3 Applications of Animal Forensics

The applications of animal forensics can be broadly viewed in the following four categories:

20.3.1 Animals Can Be the Victim

In a case report from Argentina, ranch cattle were stolen and slaughtered. The later found carcasses could be uniquely identified due to their brand (a specific farm mark on the animal coat for identification of individual animals) was used as reference sample. Their DNA profiles were compared with seized chunks of meat from a butcher's shop. The evidence (meat from butcher shop) agreed with the reference samples, so the meat could be assigned to the unambiguously killed cattle (Giovambattista et al. 2001). There are also numerous cases of cruelty to animals which include acts like bestiality and killing of animals for skin, bone, etc. In such cases a forensic scientist is expected to link traces of animal tissues available at the crime scene to the animals being killed or in case of bestiality, linking traces of sperm found in the vagina of an animal with the perpetrator or linking hairs or blood spots recovered from the perpetrator's body with the animals being assaulted.

20.3.2 Animals Can Be the Perpetrator

Animals can be perpetrators as well as victims and can be involved in an attack on a person or other animal. Thus, identification of such animals is needed as well as the identification of an unrestrained animal causing an accident or being responsible for property damage. Thus, again a forensic scientist is expected to link traces of animal material recovered from the crime scene to the animal involved in the act. DNA analysis was successfully used to identify a dog that had a miniature horse killed and another seriously injured. The offender animal was successfully identified with the help of traces of horse blood found at the edge of the dog's water bowl. They were consistent with the genetic profile of the dead horse (Agronis).

20.3.3 Animals Can Be the Witness

Animal DNA can link a suspect with a crime scene or victim. Transfer of DNA from hair, saliva, blood, urine, or feces can occur during the commission of a crime—from the victim's pet to the suspect or crime scene and from the suspect's pet to the victim or crime scene. In a case, the conviction of a man for the murder of a 7-year-old girl in California was supported by mitochondrial DNA analysis of dog hairs that matched a pet belonging to the victim.

20.3.4 Wildlife Forensics

Illegal trade of animals and their by-products is a growing global black market commerce. Since weight-for-weight wildlife trade is equally or more profitable than drugs or arms, and has less risk associated with it, illicit wildlife trade is attractive to criminals. It poses serious threats, both direct and indirect, to global biodiversity. A chain of events occurs which leads to the extinction of the species involved. Species sought for trade are directly affected by overexploitation. Overexploitation is fueled by the exaggerated values placed on rarer species by the black market. 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 (Courchamp et al. 2006). Overexploitation of wild populations can cause global extinction. Wildlife trade also provides avenues for the introduction of exotics with the potential to spread disease to native species (Lips et al.

2006; Pedersen et al. 2007; Skerratt et al. 2007; Smith et al. 2006; Spinks and Shaffer 2007) or to become invasive (Keller and Lodge 2007; Normile 2004; Reed 2005; Weigle et al. 2005). A specialized field of animal forensics called wildlife forensics deals with such cases. Wildlife DNA forensics is an applied field that has emerged from a synthesis of conservation genetic research and forensic genetic practice to meet the increasing need for investigative tools in wildlife law enforcement. The ultimate objective of such investigative tools is identification of evidence items in order to determine the species, population, relationship, or individual identity of a sample.

At the heart of all this lies the challenge of establishing a relationship or individual identity of a degraded, compromised, and trace sample recovered from the crime scene. Since all living things contain DNA and all DNA exhibit variability both among and within species, any biological material associated with a legal case carries in it information about its source. Moreover, since techniques have been developed for extracting DNA even from traces of almost all sorts of samples, DNA forensics has evolved to become an indispensable tool of modern animal forensic science.

20.4 Forensic Genetic Identification Methods

Molecular animal forensics employs various genetic tools, for example, DNA sequencing, single nucleotide polymorphism (SNP), PCR-RFLP, and microsatellite analysis, to characterize or identify a sample recovered from crime scene or illegal wildlife traders and black markets involved in wildlife trade. The genetic identification can be done at three levels: species identification, identification of geographic origin, and individual identification.

20.4.1 Species Identification

To identify the species of an evidence sample using genetic analysis is the most common application in wildlife DNA forensics and is of lesser use in human forensics. Species identification is used in cases of illegal poaching in order to identify trace evidence in the field or from a suspect's possessions (Gupta et al. 2005). It has a particular use in the identification of traded products that have lost identifying morphological characters, such as processed wood (Deguilloux et al. 2002), traditional medicines (TMs) (Hsieh et al. 2003; Wetton et al. 2004; Peppin et al. 2008), and shark fins (Shivji et al. 2002; Chapman et al. 2003).

Genetic species identification relies on the isolation and analysis of various DNA markers present in eukaryotic genome such that they show variation among species but are generally conserved within species. There are two types of genetic markers: mitochondrial and nuclear markers.

20.4.1.1 Mitochondrial Markers

These include:

20.4.1.1.1 Mitochondrial Ribosomal RNA Markers

There are two ribosomal RNA (rRNA) genes, 12s rDNA and 16s rDNA, present inside animal mitochondria. Out of these 12s rDNA is highly conserved and is used to understand the genetic diversity of higher categorical levels, phyla and subphyla, while 16s rDNA is often used for studies at middle categorical levels, families or genera (Gerber et al. 2001). For molecular analysis, conserved primers are used to amplify these markers, and the amplicons are then sequenced. Sequencing data are then aligned and compared using appropriate bioinformatics tools. In a study, Turan (2008) used mitochondrial 16s rRNA to elucidate the pattern of relationships and systematic status of 4 genera, including nine species of skates living in the Mediterranean and Black Seas (Turan 2008).

20.4.1.1.2 Mitochondrial Protein-Coding Gene Markers

There are 13 protein-coding genes present in animal mitochondria. Compared to ribosomal RNA genes, mitochondrial protein-coding genes have faster evolutionary rates and are relatively conserved. Therefore, they are regarded as powerful markers for genetic diversity analysis at lower categorical levels, including families, genera, and

species. Mitochondrial protein-coding genes that have been used for molecular analysis include cytochrome b (cyt b), NADH dehydrogenase subunit 5 (318 bp), and cytochrome oxidase I (COI) gene. However, cytochrome b is one of the most extensively used protein-coding genes of the mitochondrial genome. Its sequences have been used to understand the genetic diversity for better conservation management of Tibetan gazelle (*Procapra picticaudata*), a threatened species on the Qinghai-Tibet Plateau of China (Zhang and Jiang 2006). Similarly, NADH dehydrogenase subunit 5 (318 bp) has been used for phylogenetic analysis of multiple individuals of different species from the Felidae family, where it successfully differentiated eight clades reflecting separate monophyletic evolutionary radiations (Johnson and O'Brien 1997). Mitochondrial cytochrome oxidase I (COI) gene is most commonly used for developing DNA barcodes for species identification and biodiversity analysis. It has been used to reveal unrecognized species in several animal groups. DNA barcoding is a process in which one or a few genes are selected such that they are shared by most, if not all, organisms on earth and which show large interspecific but small intraspecific levels of variation. Such gene sequences can then be regarded as equivalent of species-specific barcodes.

A species identification method for 13 animal species, 9 of which were wild (wildebeest, zebra, Thomson's gazelle, impala, reedbuck, kongoni, oryx, warthog, and hippopotamus) and four were domestic (bovine, caprine, ovine, and porcine), was developed using a mitochondrial DNA marker in polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP) analyses using *Rsa*I enzyme. A 700-bp region of mitochondrial cytochrome b gene/control (D-loop) was successfully amplified from all the samples, and a unique RFLP profile specific to each species was generated using *Rsa*I enzyme (Malisa et al. 2005).

20.4.1.1.3 Mitochondrial Control Region Markers

There is a noncoding region termed the control region (CR or D-loop) present in mitochondrial DNA. It has a role in replication and transcription of mitochondrial DNA and is approximately 1 kb in size. The D-loop segment exhibits comparatively higher level of variation than protein-coding sequences and is therefore used for identification of species and subspecies.

The decreasing order of conserved sequences in various mitochondrial DNA markers is 12S rDNA > 16S rDNA > cytochrome b > control region (CR or D-loop).

20.4.1.2 Nuclear Markers

Various nuclear markers used for DNA fingerprinting include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites. All these markers are described below in detail under different sections.

However, the most commonly used markers in animals are mitochondrial DNA markers particularly cytochrome b (Parson et al. 2000) and cytochrome oxidase subunit I (COI) (Hebert et al. 2003a, b), as their mutation rates roughly coincide with the rate of species evolution. Moreover, in comparison to nuclear DNA, where in diploid species a single nucleus carries just two copies of each marker, mitochondrial DNA is present in multiple copies within a single cell, and so the mitochondrial DNA markers are present in multiple copies. Thus, the chances of success increase significantly when analyzing trace evidence samples that typically contain relatively little cellular material (Butler and Levin 1998; Budowle et al. 2003). However, it is recommended that multiple genes, preferably both mitochondrial and nuclear genes, are used for species delimitation (Brower et al. 1996; Maddison 1997; Page 2000; Sites et al. 1996).

Various techniques used in species identification are described below:

20.4.1.2.1 DNA Nucleotide Sequencing

It is a primary method of species identification and usually involves sequencing around 500 bases of DNA to provide a species-specific sequence. Sequencing identifies each nucleotide (base) within a specific target region of DNA (the genetic marker). DNA nucleotide sequencing is particularly advan-tageous because it enables the designing

of universal PCR primers that can be used for the amplification of DNA from a wide range of species without any prior information regarding the sample (Verma and Singh 2003). It also enables the designing of species-specific primers so that more than one species can be identified in a single multiplex PCR reaction. Furthermore, sequencing provides data for developing genetic markers such as single nucleotide polymorphisms (SNPs) and microsatellites, which describe specific areas of sequence variation.

Various evidence samples are processed, and DNA is isolated as already described above. After determining the nucleotide sequence of this unknown DNA, its identification is done through the use of a reference database search whereby the unknown sequence is compared to those of known samples: a measure of the similarity between sequences is calculated, and the most similar species is attributed to the sample (Parson et al. 2000; Branicki et al. 2003). Therefore, it is necessary to have a well representation of that species in the public databases. Closely related species may have sequence similarities of 90–95 % or higher. Moreover, since the level of similarity also depends on the total length of the sequence which is being matched, it is ultimately left to the experience and judgment of the forensic scientist to evaluate the strength of evidence when undertaking a sequence similarity match.

We can also construct a phylogenetic tree and determine the evolutionary relationships between the test sample and reference sequences from each possible species. The position of the test sample in the tree allows the closest reference species to be identified as the likely source (Avise 1994; Roman and Bowen 2000; Verma et al. 2003).

20.4.1.2.2 SNP Typing or Genotyping

We can go for either full DNA sequencing or sequence only certain regions of the genome that contain various genetic markers which are species specific. The differences observed among species at a genetic marker are largely due to single base pair changes in the DNA sequence, known as single nucleotide polymorphisms (SNPs). Therefore, SNP typing, or genotyping, allows specific variable sites in a DNA sequence to be investigated enabling species differentiation through faster and cheaper tests that do not require such long fragments of high-quality DNA as is required in DNA sequencing methods. However, in comparison to DNA sequencing, lesser information is gained in SNP typing.

SNP typing requires shorter DNA sequences and thus minimizes the size of the genetic marker targeted. This is often necessary to obtain results from samples that either are degraded or have been highly processed, fragmenting the DNA (Butler et al. 2003; Hajibabaei et al. 2006). However, there is also an increased risk of sample misidentification associated with SNP typing. This must be considered when designing assays and interpreting forensic data.

There are multiple methods for typing SNP markers, some of which are described below.

PCR-RFLP

In this technique the DNA segment of interest is amplified using PCR and is then subjected to digestion by restriction enzymes. Restriction enzymes are a special category of enzymes that recognize specific base pair sequence motifs (that are often mirror images) and cut the amplified fragment at these sites. Species that differ in nucleotide composition at the restriction enzyme recognition sites will differ in whether or not the enzyme cuts the DNA or in the position at which the enzymes cut the sequences. This generates DNA fragments of differing lengths (i.e., polymorphic fragments), in which the number and size of the fragments depend on the number of cutting sites in the DNA fragment of interest (Upholt 1977). Electrophoresis of the digested DNA samples thus reveals characteristic banding patterns in different taxa. Such a banding pattern becomes a DNA fingerprint of that taxa or species and is termed as RFLP profile. Similarly, in cases where restriction enzyme sites coincide with an SNP marker, a different RFLP profile is generated in different species. However, selection of restriction enzymes for PCR-RFLP analysis must ensure that the variability between species is appropriately represented so that all species tested can be accurately discriminated from each other by their unique RFLP profile.

Using primers 5′GTGCTACGAAAGCAGG3′ and 5′GGCGCGGATACTTGCATGTG3′, specific amplification of mitochondrial DNA D-loop

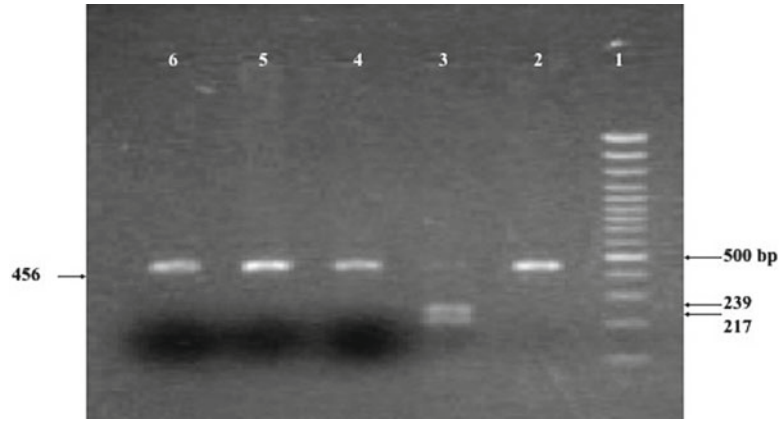

Fig. 20.2 Species specific gene amplification of different animal species; 100-bp ladder (lane 1), cattle (lane 2), buffalo (lane 3), goat (lane 4), sheep (lane 5), and nilgai (lane 6)

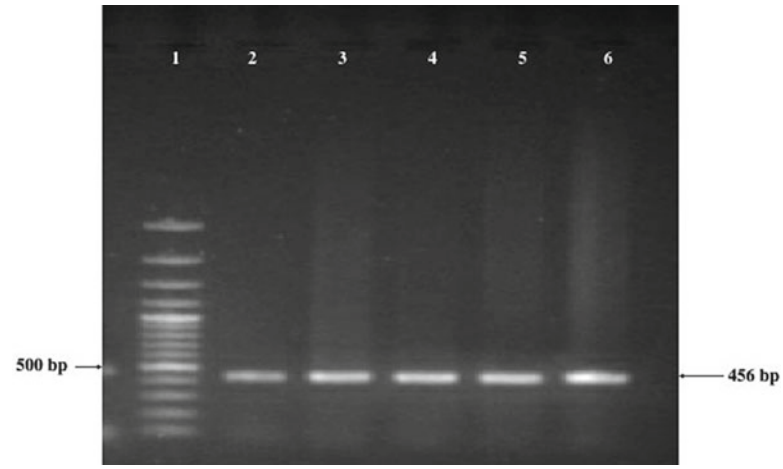

Fig. 20.3 Restriction enzyme digestion of species-specific PCR products using *Hha*1; 100-bp ladder (lane 1), cattle (lane 2), buffalo (lane 3), sheep (lane 4), goat (lane 5), and nilgai (lane 6)

region was carried out followed by restriction analysis of amplicons with *Dra*I endonuclease, and a unique RFLP profile was generated for wild boar and domestic swine, offering a useful tool to reveal fraud in meat substitutions as well as in legal cases to verify wild boar meat (Samaraweera et al. 2011). In order to differentiate between five different species (cattle, buffalo, sheep, goat, and nilgai), cytochrome b gene-specific primer pair (primer pair-D) was designed which successfully amplified a 456-bp fragment of cytochrome b gene in all the five species (Fig. 20.2). RFLP analysis of this fragment of cytochrome b gene was done using restriction enzymes Hha1, Alu1, and Apo1, and a unique RFLP profile was generated for each of the different species (Figs. 20.3 and 20.4) (Prasad et al. 2008; unpublished data). Moreover, the primer pair was shown to be very sensitive and could successfully amplify DNA extracted from boiled and processed tissue samples (Fig. 20.5). A patent was awarded to the authors for this study.

Allele-Specific PCR

Primers used in PCR for amplifying genetic markers can be designed for regions where DNA

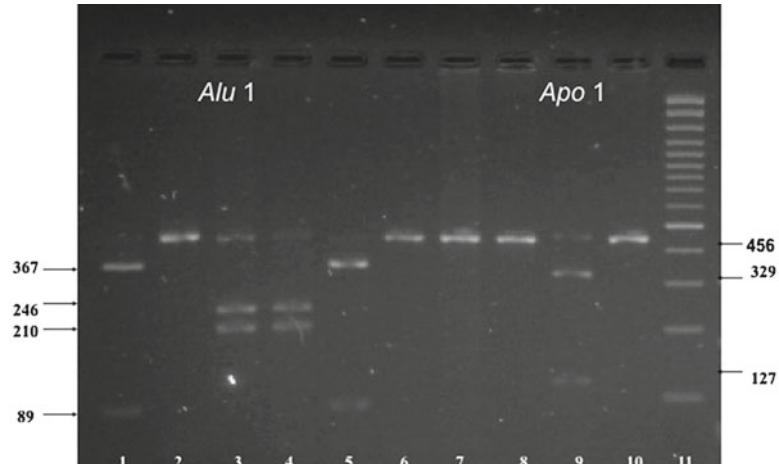

Fig. 20.4 Restriction digestion of species-specific PCR products using Alu 1 and Apo 1; cattle (lane 1, 6), buffalo (lane 2, 7), goat (lane 3, 8), sheep (lane 4, 9), nilgai (lane 5, 10), and 100-bp ladder (lane 11)

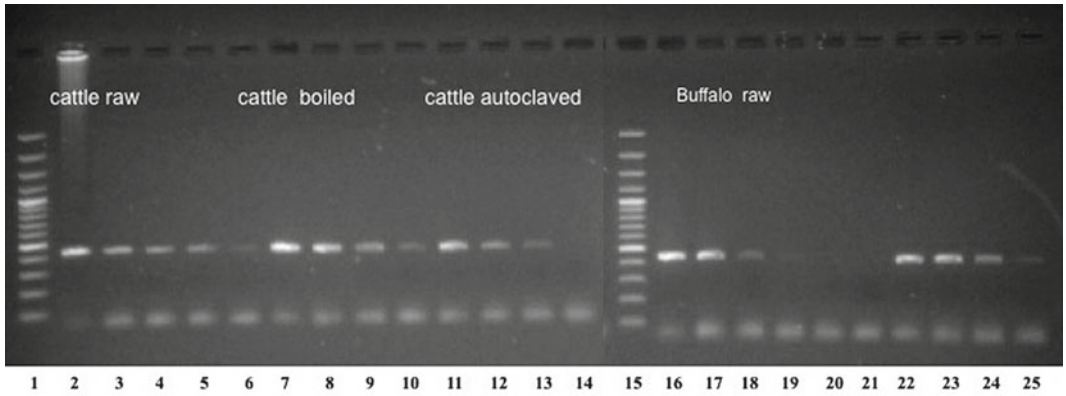

Fig. 20.5 Species-specific gene amplification of raw and processed tissue samples of cattle and buffalo; cattle raw DNA 0.3 ng (lane 6), cattle boiled DNA 0.5 ng (lane 10), cattle autoclaved DNA 2 ng (lane 13), buffalo raw DNA 3.4 ng (lane 18), and buffalo autoclaved DNA 10 ng (lane 24) were amplified

varies between species and populations. Such primers are known as allele-specific primers. SNP is responsible for generation of different alleles of a genetic marker in a population or species. Allele-specific primers are designed so that nucleotide sequence of the primers includes the SNP site, and that PCR only works when DNA from the target sequence is present in a sample. Thus, when such primers are used in PCR for amplifying unknown DNA samples, amplification of only that allele of a genetic marker occurs which has a sequence complementary to the allele-specific primers. However, for the development of species-specific primers, it is necessary to consider sequence data from all species likely to be encountered, for the design of putative primers. For example, species-specific primers that amplify the nuclear ITS2 region and the mitochondrial cytochrome b have been used to develop assays for the identification of various shark species from dried fins or meat (Moore et al. 2003; Clarke et al. 2006; Magnussen et al. 2007; Pank et al. 2001; Shivji et al. 2005).

Species-specific primer pairs based on mitochondrial D-loop and 12S ribosomal ribonucleic acid (rRNA) gene were designed that successfully

amplified 629- and 322-bp DNA fragments, respectively, from the DNA sample extracted from pig meat. Therefore, a highly specific single-step polymerase chain reaction (PCR) was optimized which provides a valuable tool for identification of pig meat and to avoid its fraudulent substitution and adulteration (Arun et al. 2012).

20.4.1.2.3 Multiplex PCR

Species-specific primers have an advantage that they can be used in a multiplex PCR which is a more robust technique for species identification. Here several primers are added to a PCR to simultaneously amplify different DNA regions of the target species in a single PCR reaction. However, universal primers that amplify across all potential taxa should be included as a control in the multiplex assay. This reduces the incidence of false negatives. If the universal primer amplifies but the species-specific primers fail to amplify, it can be confirmed that target species is absent in the sample. On the other hand, if both the universal and species-specific primers fail to amplify, then there is a problem with PCR reaction, and so the result is inconclusive. If more than one species-specific primers are used in a multiplex PCR reaction, several different species can be identified in a single assay. For example, in a study, Shivji et al. (2005) used six different species-specific primers and two universal shark primers (for positive controls) to identify six species of sharks commonly encountered in North Atlantic fisheries (Shivji et al. 2005).

Allele-Specific Probes

In this case instead of allele-specific primers, universal primers are used in combination with a specific probe. Universal primers are the primers that are designed for conserved regions of DNA and are used in PCR for amplifying genetic markers. Thus, when such primers are used in PCR for amplifying unknown DNA samples, amplification of all the alleles of a genetic marker occurs. However, subsequent use of different probes that are designed to attach to different DNA sequence variants allows the base present at a SNP site to be detected.

Even though, SNP typing methods are applied in the context of species detection, rather than species identification, they have the advantage of allowing the analysis of samples where multiple species are present. A more powerful method of species identification can be designed by combining the power of DNA sequencing and SNP typing. By using species-specific PCR primers, a single DNA sequence for the target species is generated from a sample containing multiple species DNA. This sequence can then be identified to species level to categorically demonstrate its presence in the sample.

20.4.1.2.4 Random Amplified Polymorphic DNA (RAPD)

It is a simple and least expensive technique. In this technique arbitrary primers which are usually ten bases long are used to amplify random segments of DNA. No prior information about the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. If the sample DNA has in it sequences complimentary to the primer oligonucleotide, PCR products will be detected, but if the sample DNA does not have the complimentary sequences, no product will be detected. Hence, random primers will or will not amplify a segment of DNA, depending on positions that are complementary to the nucleotide sequence of the primers. For example, no amplification occurs if primers anneal too far apart or 3′ ends of the primers are not facing each other. Therefore, in this technique we have to keep on using different random primers until we hit a pair of random primers that give a unique DNA profile for each DNA sample. Since the technique relies on a large, intact DNA template sequence, it cannot be used in case of degraded DNA samples.

Alternatively, bands which are specific to the target species are identified from the DNA fingerprint, extracted, and sequenced. These sequences are then used to design primers that will specifically amplify the species-specific region, termed a sequence characterized amplified region (*SCAR*). Subsequently, these primers can be used

for rapid identification of species. SCAR method is a highly reproducible technique.

20.4.1.2.5 Amplified Fragment Length Polymorphism (AFLP)

This technique combines some features of both RFLP and RAPD analyses and has greater differentiation power than RAPD. It is a PCR-based tool and uses restriction-enzyme-digested fragment as template for PCR amplification using primers that are complementary to the adaptor sequence, the restriction site sequence, and a few nucleotides inside the restriction site fragments. The amplified fragments are then visualized on denaturing polyacrylamide gels.

In practice, suppose we have two DNA samples. Digest each genomic DNA with a combination of two restriction enzymes, commonly *EcoR*1 and *Mse*1 for animals. Double-stranded *EcoR*1- and *Mse*1-specific linkers are then ligated to the fragment ends in both the samples. A pre-selective amplification of *EcoR*1/*Mse*1 templates is carried out using primers that anneal to the linker ends of the fragments and have one selection nucleotide at their 3′ end. A final selective PCR amplification step is carried out in which additional selection nucleotides are added at the end of the *EcoR*1- and *Mse*1-selective primers, and the primers are fluorescently labeled. Fragments are separated by migration through a polyacrylamide gel and detected by a laser as the fragments migrate through the gel. In this way a DNA profile is created for both the samples which can then be analyzed. If the two samples belong to the same species, they will have a similar DNA profile. AFLPs arise due to a difference in restriction sites in the samples, mutations beyond the restriction sites that affect complementarity with the selection nucleotides of the AFLP primers, or due to deletions and insertions within the amplified restriction fragments. Like RAPD, this technique has an advantage that no prior sequence information is needed.

20.4.1.2.6 Microsatellite Genotyping

This technique is more robust than SNP genotyping and RAPD and AFLP analyses because of hypervariability of microsatellite DNA markers. For the purpose of species identification, those microsatellite markers are chosen that show a considerable variation among species but are generally conserved within species. If on the other hand the markers show considerable variation within species or populations, the technique can be effectively used for individual identification. This technique is discussed in detail in the section describing individual identification.

20.4.2 Identification of Geographic Origin

Identification of geographic origin has applications almost only in wildlife forensics rather than in human forensics. In order to overcome a mismatch between wildlife legislation, which usually operates within political boundaries, and species distributions, which are governed by biological and environmental factors, wildlife crime investigations often seek for answers to questions concerning the geographic origin of a sample. For example, to enforce CITES regulations, the need to determine the geographic source of a specific sample, in addition to identifying the species, cannot be ruled out. Similarly, the effective management of marine-protected areas requires that illegally harvested stocks be distinguished from those taken legally from elsewhere. Returning lost or captured animals to their native geographic area after genotypically assigning them to that population reduces the risk of outbreeding depression. The possibility of corruption of the evolutionary processes leading to divergence among geographic isolates can also be minimized by such targeted releases.

When it comes to forensic science, identifying the geographic origin means identifying its reproductive population of origin. Since populations are most often delineated by geographic rather than reproductive barriers, they are often capable of sharing genetic material. Therefore, in comparison to species identification, DNA markers are less likely to show discrete differences among different populations. Geographic origin identification thus requires the source population to be sufficiently genetically distinct from other candidate

populations and heavily relies on the existence of population data from multiple areas. However, when populations are so isolated from one another that there is effectively no exchange of genetic material between them, genetic differences gradually accumulate over evolutionary time to a point where members of an isolated region share the same types of genetic marker (alleles) within their population but exhibit different alleles to that of any other population. Such markers can be effectively used for identifying populations or assigning geographic origin to a sample.

Geographic origins of an individual can be identified if there is known genetic structure within the region of interest using the following methods:

20.4.2.1 Phylogeography

This method assesses the geographic distribution of genealogical lineages where specific mtDNA haplotypes are associated with broad geographic regions (Avise et al. 1987). For example, hypervariable mtDNA control region, or D-loop, is often used as a marker in geographic origin identification with individual control region sequence types (haplotypes) corresponding to specific populations.

20.4.2.2 Population Assignment Methods

When sufficient mitochondrial DNA variation is absent, various genetic markers from the nuclear genome that show variability among regions can be used for the identification of geographic origin. These allelic differences at hypervariable nuclear DNA genetic markers between groups of individuals (populations) form the basis of population assignment methods. The hypervariable markers most often used in such methods are AFLPs (amplified fragment length polymorphisms) and microsatellites (also called short tandem repeats or STRs). Although these markers do show discrete differences, individual alleles are often distributed across populations. Therefore, differentiation can only be achieved on the basis of differing allele frequencies. The frequency of the alleles observed in a population can be used to characterize its genetic structure and to assess the probability of a sample originating from that area. Therefore, population assignment methods heavily rely on the development of large genetic databases that provide representative allele frequencies for all of the potential source populations and also on the use of statistical analysis to provide quantitative probabilities of assignment for an unknown sample to each of those populations.

20.4.3 Individual Identification, Sexing, and Parentage

20.4.3.1 Individual Identification

The use of DNA profiling for the individual identification of genetic evidence is of particular importance in human forensics and is considerably lesser relevant to animal and wildlife forensics, where its main applications include identification of stolen animals and the authentication of legally traded wildlife products or in cases of poaching, where it may be necessary to demonstrate that a horn, tusk, bone, or skin has originated from a specific individual. It has over the past 20 years revolutionized human forensic analysis.

Those genetic markers that are highly variable within species are likely to show differences among individuals. By targeting such markers through DNA profiling studies, a unique DNA profile for each specific individual can be arrived at. Such a DNA profile becomes the DNA fingerprint of that individual and can be used for individual identification.

Various methods of individual identification, sexing, and parentage are described below.

20.4.3.1.1 Microsatellite Genotyping

This technique is best suited for parentage analysis. Microsatellites are hypervariable markers, also known as short tandem repeats (STRs) or simple sequence repeats (SSRs). Microsatellites are short sequence motifs consisting of two, three, or four nucleotides and can be repeated 3–100 times. Microsatellites have a high mutation rate predominantly due to slippage of the polymerase during DNA replication. The longer the loci, the more will be the alleles due to the greater potential for slippage. Consequently, the greater the

number of markers used, the lesser are the chances that another individual has the same series of alleles (same profile). One common example of a microsatellite is a $(CA)_n$ repeat, where n varies between alleles. These repeats are frequent in human and other genomes and are present every few thousand base pairs.

Microsatellites are codominant markers with bi-allelic or multi-allelic presentation in an individual or a population, respectively. Gene variants or alleles are inherited from both parents. These alleles are amplified in a PCR reaction and visualized on a polyacrylamide gel. Homozygote individuals will have the same-sized STR repeats, whereas heterozygote individuals will have different-sized repeats. Since at a particular microsatellite locus there are often many alleles present, it becomes possible to identify the progenitor of a particular allele within pedigrees. Therefore, microsatellites are also ideal for determining paternity.

For individual identification, DNA profiles are produced for different samples and thereby analyzed. If two samples produce different DNA profiles, it means that the samples did not originate from the same individual. On the other hand, if the samples share the same profile, there is a significant possibility that the samples are from the same individual. However, it becomes necessary to calculate the probability that the two individuals have the same profile by chance. Various factors which affect this probability include the number and variability of markers in the profile, the frequency of the alleles in the species, and the degree of relationship between the individuals in the population from where the samples were taken. Therefore, it becomes necessary to have representative sample of DNA profiles from the population. The genetic profiles can be generated using information from the mitochondrial control region, sex determination markers, or microsatellite loci.

In case of wildlife forensics, there are situations where animals are sold as meat or highly processed products. Identification of an individual based on their unique genetic profile has a particular application in such cases where it is used to monitor the number of animals entering commercial markets. Similarly, in order to distinguish between legally and illegally obtained specimens, individual identification is needed. However, it is necessary to maintain a DNA register where each legal specimen is DNA profiled in a certified laboratory and the profiles are lodged in a database.

20.4.3.1.2 DNA Nucleotide Sequencing, SNP Typing, RAPD, and AFLP

As with species identification or identification of geographic origin, individual identification and paternity testing can also be done by SNP typing, RAPD, AFLP, and DNA nucleotide sequencing, using both nuclear and mitochondrial DNA markers. However, here it is important that the genetic markers have a considerable variability within species or populations so that a unique DNA profile can be assigned to each individual.

20.4.3.2 Sexing

There are situations where it becomes necessary to determine the sex of an individual. For example, in case of immature animals in which secondary sexual characters have not developed, sexing on the basis of morphological characters becomes very difficult. In case of Asian elephants where tusks are only present in males, drastic declines in the numbers of males from hunting for their ivory can result in unbalanced sex ratios. Similarly, monitoring the trade in some countries where qualified hunters are restricted to hunting only one sex requires determination of the sex of the animals hunted.

In animals that have a heteromorphic sex chromosome system, molecular sexing relies on either the PCR amplification of fragments specific to the Y or W chromosomes or the amplification of homologous fragments from both sex chromosomes. The former approach can be used in placental mammals and marsupials, in which amplification of the Y-specific SRY locus is expected only in males. In the latter approach, amplification of homologous fragments from both sex chromosomes such as ZFX and ZFY genes is carried out using the same pair of PCR primers and later differentiated on the basis of polymorphism between the two fragments using various techniques like PCR-RFLP.

Sex-specific molecular markers, however, are not universal and need to be developed independently for different classes of organisms. For example, in lizards *Calotes versicolor*, even though SRY gene is present in males, it is also present in 50 % of females.

Various sex-specific genes that have been extensively used in sex determination include:

20.4.3.2.1 SRY Gene

Also known as sex-determining region Y gene, it is a sex-determining gene present on the Y chromosome in placental mammals and marsupials and initiates male sex determination. It can be detected in males by nucleic acid hybridization or by PCR amplification using specific primers. However, it is important to include an appropriate control, usually another nuclear gene such as actin, in the PCR reaction to avoid generation of false females in case failure of amplification is because of technical reasons.

In a study Gowans et al. (2000) used primers developed for the sperm whale SRY gene and amplified a 147-bp fragment of the SRY gene from tissue samples taken by harpoon biopsy from live northern bottlenose whales and provided an accurate sexing procedure for these animals (Gowans et al. 2000). An inexpensive test was developed based on the SRY gene on the Y chromosome for identifying male Asian elephants from poached carcasses (Gupta et al. 2006).

20.4.3.2.2 ZFY and ZFX Genes

ZFY gene encodes a zinc finger-containing protein known as zinc finger Y-chromosomal protein that functions as a transcription factor, whereas ZFX gene encodes zinc finger X-chromosomal protein. Therefore, ZFY is a Y-chromosome-linked gene, and ZFX is an X-chromosome-linked gene.

In a study Fontanesi et al. (2008) developed a sexing method for three leporid species (*Oryctolagus cuniculus*, *Lepus europaeus,* and *Lepus timidus*) based on the analysis by PCR-RFLP of point mutations that differentiate the ZFX and ZFY gene sequences. A 432-bp fragment of exon 11 of the ZFX and ZFY gene was amplified using a same set of primers, and restriction enzyme digestion was carried out using different enzymes which generated a separate RFLP profile for male and female DNA samples (Fontanesi et al. 2008).

In another study, Malisa et al. (2005) successfully identified the sex of 13 animal species, 9 of which were wild (wildebeest, zebra, Thomson's gazelle, impala, reedbuck, kongoni, oryx, warthog, and hippopotamus) and 4 were domestic (bovine, caprine, ovine, and porcine), following successful amplification of gender-specific, SRY and ZFY/ZFX, chromosomal domains using sex-specific primers (Malisa et al. 2005).

20.4.3.2.3 Chromobox-Helicase-DNA-Binding Gene (CHD1)

In case of avians, sex-specific markers, one on the Z chromosome and one on the W chromosome, can be used successfully for gender determination because avian males are homozygotes (ZZ), whereas females are heterozygotes (ZW). For example, in birds other than some flightless species (ratites), chromobox-helicase-DNA-binding gene is localized on the W (female-specific) chromosome (CHD1W), although it has a homologue (CHD1Z) on the Z chromosome. PCR primers were designed to amplify the intron of the CHD1 gene, and fragments were generated which consistently differ in size between the W and Z versions (Griffiths et al. 1998).

20.4.3.3 Parentage

Genetic markers are inherited from one generation to the next. Therefore, DNA profiles can be used to verify parent-offspring relationships. Various genetic markers, for example, microsatellites, that are used for individual identification can also be used for parentage verification analysis. In order to confirm a particular parent-offspring relationship, the alleles present in the DNA profile of an individual must also be present in its putative parents, one allele per marker in each parent. In case alleles observed in an individual do not correspond to those in the putative parental profiles, then the possibility of the individual being their offspring can be excluded.

20.5 Conclusion

With the discovery of newer genetic markers, production of more reference databases, validation of laboratory techniques, development of more

efficient protocols for sample recovery and DNA extraction, and the ability to type samples of low quantity and quality, DNA analysis is going to revolutionize animal DNA forensics. Genome sequencing is becoming faster and less expensive, paving the way for the development of novel markers for forensic identification and the subsequent production of reference data. The increase in genomic data generated through high-throughput sequencing technologies will enable the discovery and application of markers associated with adaptive traits, thereby allowing greater resolution of geographic population identification. It will also provide universal nuclear primers that can amplify informative regions over a broad range of taxa.

So long as the emerging technologies will keep on adding to the forensic genetic toolbox, current genetic technologies, with the help of various nuclear and mitochondrial DNA markers, are capable of addressing most forensic questions. The choice of a genetic marker depends on the forensic question to be addressed and the ecology and genetic knowledge of the species. However, in order to validate their routine use for forensic application and admissibility as evidence, further research needs to be done. Furthermore, it is necessary to establish a network of accredited animal and wildlife DNA forensic laboratories.

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