Molecular identification of animal species using Polymerase Chain Reaction based techniques

Thesis

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By

NAGAPPA KARABASANAVAR
B.V.Sc. & A.H., M.V.Sc
(Veterinary Public Health)

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Pantnagar *(Nagappa Karabasanaavr)*
June, 2008 *Author*

G. B. Pant University of Agriculture & Technology

Dr. S.P. Singh
Professor & Head

Department of Veterinary Public Health
College of Veterinary & Animal Sciences
G. B. Pant University of Agri. & Tech.
Pantnagar - 263145, (Uttarakhand)
INDIA

CERTIFICATE

This is to certify that the thesis entitled "Molecular identification of animal species using Polymerase Chain Reaction based techniques", submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with major in Veterinary Public Health and minor in Biochemistry and Animal Biotechnology, of the college of Post-Graduate Studies, G. B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona fide* research carried out by Dr. Nagappa Karabasanavar, Id. No. 29840, under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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Pantnagar
June, 2008

(S.P. Singh)
Chairman
Advisory Committee

G.B. Pant University of Agriculture & Technology
Pantnagar- 263 145
Distt. - U.S. Nagar, Uttarakhand, INDIA

CERTIFICATE

We, the undersigned, members of the Advisory Committee of Dr. Nagappa Karabasanavar, Id. No. 29840, a candidate for the degree of Doctor of Philosophy, with major in Veterinary Public Health, agree that the thesis entitled "Molecular identification of animal species using Polymerase Chain Reaction based techniques" may be submitted in partial fulfillment of the requirements for the degree.

Dr. S.P. Singh
Prof. & Head, Veterinary Public Health
(Chairman)

Dr. Mahesh Kumar
Prof. & Head,
Veterinary Epidemiology &
Preventive Medicine
(Member)

Dr. V.P. Dixit
Prof., Vety. Public Health
(Member)

Dr. Sanjeev Agrawal
Prof. & Head, Biochemistry
(Member)

Dr. Umapathi, V
Asst. Prof., Vety. Biochemistry
(Member)

List of Abbreviations

%	:	Per cent
µg	:	Micro gram
µl	:	Micro liter
@	:	At the rate of
AFLP	:	Amplified Fragment Length Polymorphism
AP-PCR	:	Arbitrarily Primed Polymerase Chain Reaction
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
BSE	:	Bovine spongiform encephalopathy
CJD	:	Creutzfeldt Jacob Disease
cm	:	Centimeter
CNS	:	Central Nervous System
Ct	:	Threshold Cycle
Cyt	:	Cytochrome
DALP	:	Direct Amplification of Length Polymorphisms
dATP	:	Deoxy Adenosine triphosphate
dCTP	:	Deoxy Cytosine triphosphate
dGTP	:	Deoxy Guanine triphosphate
DNA	:	Deoxy ribonucleic acid
DNAase	:	Deoxyribonuclease
dNTP	:	Deoxy nucleotide triphosphate
dsDNA	:	Double stranded DNA
dTTP	:	Deoxy Thymine triphosphate
EC	:	European Commission
EDTA	:	Ethylene diamine tetra acetate
et al.,	:	*et alii* (and others)
etc	:	Et cetera
Fig	:	Figure
FINS	:	Forensically Informative Nucleotide Sequencing
fm	:	Femtogram
g	:	Gram (s)
GFAP	:	Glial Fibrillary Acidic Protein
GH	:	Growth Hormone
h	:	Hour (s)
i.e.	:	that is
ITS	:	Intervening Transcribed Spacer
kb	:	Kilo base
Kg	:	Kilogram (s)
LINE	:	Long Interspersed Nuclear Elements
M	:	Molar
MBM	:	Meat and Bone-Meal
mg	:	Milligram (s)
min	:	Minute (s)
ml	:	Milliliter (s)
mM	:	Milli molar

mRNA	:	Messenger RNA
mt	:	Mitochondria (l)
mt DNA	:	Mitochondrial Deoxy ribonucleic acid
MW	:	Molecular weight
NFW	:	Nuclease Free Water
ng	:	Nano gram (s)
nm	:	Nano meter
O.D.	:	Optical Density
^{o}C	:	Degree Centigrade
p.m. (p mol)	:	Pico moles
PCR	:	Polymerase Chain Reaction
pg	:	Pico gram
ppm	:	Parts Per Million
RAPD	:	Random Amplified Polymorphic DNA
RE	:	Restriction enzyme
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic acid
RNase	:	Riboendonuclease
rpm	:	Revolutions per minute
s	:	Second (s)
SDS-PAGE	:	Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis
SINE	:	Short Interspersed Nuclear Elements
SSCP	:	Single Strand Conformation Polymorphism
SWAPP	:	Sequencing With Arbitrary Primer Pairs
TAE	:	Tris Acetate EDTA
TBA	:	Tris Borate EDTA
TE	:	Tris EDTA
TSE	:	Transmissible Spongiform Encephalopathies
UV	:	Ultra Violet
v	:	Volume
V	:	Volts
vCJD	:	Variant Creutzfeldt Jacob Disease
viz.,	:	That is to say
w/v	:	Weight / volume

Contents

1 Introduction

There has been unprecedented growth in the food industry consequent to the growing demand for quality food by an ever increasing population. Nowadays, consumers have become health conscious and thus demand quality food. The adulterated food however, often enters the supply chain and jeopardizes the sentiments as well as health of the people. Substantial proportion of population has got religious considerations towards the consumption of meat of a particular animal species and any deviation may result into detraction from its consumption. In addition, the presence of animal derived material in the vegetarian diet at times also creates havoc in the conscious society. Hence, the meat adulteration has got social, religious, economic and public health concerns. The legal enforcement also restricts consumption of meat of wild animal species. In view of all these catastrophes that often challenge the analyst, there is an urgent need to have reliable techniques which may aid in the authentic identification of meat species. Although, numerous analytical methodologies viz. anatomical, histological, microscopic, organoleptic, chemical, electrophoretic, chromatographic and immunological techniques have been employed but none of these are considered to be complete for the purpose of species identification.

The traditional methods such as anatomical, chemical, electrophoretic, chromatographic and immunologic techniques have one or the other limitations. These procedures are either cumbersome or lack repeatability and reproducibility. For instance, immunoassays fail to differentiate the closely related species as the protein markers may possess similar isotypes in different species of animals and different components of the same animal species could behave differently making the judgment difficult. Further, techniques based on the variation in the protein profile between species could not be used for the processed, stored and communited meat and meat product samples.

Recently, DNA based molecular techniques have become popular and could provide satisfactory solution to the problem of identification of meats of different animal species. Targeting DNA for the animal speciation offers many advantages over the protein-based techniques. The DNA molecule is more stable and the composition of DNA is same in any cell of the individual. Hence, DNA based techniques namely Polymerase Chain Reaction (PCR) and its variants; Restriction Fragment Length Polymorphism (RFLP); Random Amplified Polymorphic DNA (RAPD) finger printing; DNA Hybridization, PCR-Sequencing; Arbitrarily Primed-PCR (AP-PCR) and PCR-Single Strand Conformation Polymorphisms (SSCP) have been employed for the identification of animal species in the recent past. Further, with the advent of Real Time PCR, there has been a major breakthrough in the quantitative analysis of meats of different animal species. The PCR-based techniques provide high level of specificity, sensitivity, accuracy and precision than the techniques hitherto being used for the purpose of animal species identification.

Keeping in view the acute need for a reliable technique for animal species identification, the present work was designed with the following objectives,

OBJECTIVES:

1. Designing of species specific primers for the identification of different species of food animals.
2. Development of species-specific PCR assays for the identification of cattle, buffalo, sheep, goat, pig and chicken.
3. Detection of adulteration in meat mixtures.
4. Validation of species-specific PCR assays for the identification of animal species in the raw as well as thermally processed meat samples.
5. Exploitation of these techniques for the identification of some other animal species.

2 Review of Literature

2.1 Animal species identification

Food derived from animals is sometimes presented wrongly for gaining economic benefit. This act of misrepresentation is of serious concern and is an offense under the court of law. Ideally, the food offered to the consumers should be safe, free from adulterants and should carry proper label (LMG, 1992; LMV, 1995 and TSV, 1995). In order to keep the consumers' confidence high, the foods of animal origin should be of assured quality with absolutely authenticated origin. Further, the religious prohibitions and sentiments involved in the consumption of meat of particular animal species necessitate the application of advanced analytical methodologies for animal speciation. The problem of food allergy has been centered mainly towards table egg, milk, wheat and peanuts. The epidemiological data reveal certain food allergies in ~10-20% of the world population, manifested as an Ig-E mediated hypersensitivity (Hoffman, 1983; Burke *et al.,* 1991; Businsco and Bellanti, 1993, Tanabe *et al.,* 1996 and Platts-Mills *et al.,* 1997). Also, there has been growing evidence to suggest allergies especially to beef (73%), pork (58%) and chicken (41%) (Ayuso *et al.,* 1999) in human consumers. Further, "pork-cat syndrome" where persons may be allergic to pig and cat derived material is gaining importance in the developed countries (Sabbah *et al.,* 1994; Drouet and Sabbah, 1996; Hilger *et al.,* 1997 and Bohler *et al.,* 2001).

The *"Forensic Analysis"* of the wild animal species (Colombo *et al.,* 2004) has been found useful in the conservation of the biodiversity of endangered wild fauna. Illegal trade of wild animals has been identified and the ecosystems have been protected in many countries using speciation techniques (Hofer *et al.,* 1996; Burnett, 2000; Goodall, 2000 and Malisa *et al.,* 2006). Identification of the flesh of wild animal species mixed with the meat food intended for human consumption could help in the control of zoonoses as it could bring

infection from the sylvatic foci (Malisa *et al.,* 2006). Consequent to the emergence of new *"Zoonotic threats"*, especially the prion diseases (BSE, CJD, vCJD and nvCJD) and their link to the animal reservoirs has resulted in the prohibition of animal derived material in animal feed (LMV, 1995; TSV, 1995 and Taylor and Woodgate, 1997). Contamination of the meat from the butchers' equipment was found responsible for the occurrence of vCJD in the U.K. (Adam, 2001). Further, the foot-and-mouth disease of cloven footed animals has trade and animal health implications (Kurzenhauser, 2001 and Woodhouse *et al.*, 2001).

2.2 Meat animal speciation techniques

Numerous analytical methodologies such as anatomical/histological/microscopic, organoleptic and chemical (Plowman and Close, 1988; Meyer *et al.*, 1994; 1995; Matsunaga *et al.,* 1999a and Ilhak and Arslan, 2007), electrophoretic (Bossezon *et al.,* 1966; Kim and Shelef, 1986; Costa-Rivas and Vallejo-Cordoba, 1997; Skarpeid *et al.,* 1998; Ozgen and Ugur, 2000 and Renon *et al.,* 2003), chromatographic (Carnegie *et al.,* 1985; Taylor *et al.,* 1993; Epsinoza *et al.,* 1996; Toorop *et al.,* 1997; Ashmoor *et al.,* 1998; Epsinoza *et al.,* 1999; Czesny *et al.,* 2000 and Niederer and Bollhalder, 2001), immunological (Uhlehuth, 1901; Wiener *et al.,* 1949; Berger *et al.,* 1988; Barai *et al.,* 1992; Morales *et al.,* 1994; Hofmann, 1997; Hsieh *et al.,* 1998; Macedo-Siliva *et al.,* 2000 and Hajmeer *et al.,* 2003) and DNA-based molecular techniques (Zimmermann *et al.,* 1998) have been employed for the identification of meats of different species of animals.

Protein-based techniques (gel diffusion, IEF and immunoassays) have certain limitations, as most of the target proteins being tissue dependent bring about alteration in structure and stability during thermal processing (Guoli *et al.,* 1999 and Calvo *et al.,* 2001b). Species-specific proteins (epitopes) get destroyed during heat treatment (Kang'ethe *et al.,* 1986) and protein denaturation takes place resulting in the altered electrophoretic mobility/antigenicity. Although, antisera have been raised against heat-stable animal proteins

but these could differentiate only the distantly related species (Hayden, 1981; Kang'ethe and Gathuma, 1987 and Berger *et al.,* 1988). Most importantly, since the protein composition varies between the tissues of an individual and within the same animal species, cross-species signals in closely related species posses a problem (Hofman, 1987 and Bandman and Zdanis, 1988).

Iso-electric focusing (IEF) and immunoassays were the most widely used protein-based techniques for species identification (Patterson and Jones, 1989; Rehbein, 1990; Wintero and Thomsen, 1990; Jemmi and Schlosser, 1993 and Rehbein *et al.,* 1999). However, these techniques were not found to be highly effective in processed (heated or marinated) products as most soluble muscle proteins get degraded rapidly. Even though modified methods were available for the processed meat samples; interpretation of the electro-pherogrammes was difficult and doubtful.

Further, immunoassays failed to differentiate the closely related species as the protein markers possess the similar epitopes in different species and different components of the same species could also possess different types of markers finally making the judgment difficult (Hsieh *et al.,* 1998; Hird *et al.,* 2003; Chen *et al.,* 2004 and Pfeiffer *et al.,* 2004). In view of the protein profile variability due to the loss of biological activity and the characteristics of the cell type, the protein-based methods can not be conclusively used for the processed, stored and communited meat samples (Kang'ethe *et al.,* 1986).

However, many traditional techniques such as anatomical, chemical, electrophoretic, chromatographic and immunological methods described previously for the animal speciation have limitations of repeatability, reproducibility and sensitivity. Also they are cumbersome hence unsuitable for processed samples (Plowman and Close, 1988). Although microscopic analysis is very sensitive tool for the detection of animal products such as meat-cum-bone-

meal (MBM) in animal feed; it is time consuming and also requires very high level of expertise (Michard and Ziebal, 1999).

In view of the limitations of these conventional animal speciation techniques, there has always been a search for the most reliable, sensitive and robust techniques which could put forward conclusive judgment.

2.3 Meat animal speciation using DNA-based molecular techniques

Genomics is the scientific study of structure, function and interrelationships of individual genes and the genome in its entirety (Bazer and Spencer, 2005 and Fadiel *et al.,* 2005). This field has provided enormous information about the DNA sequences starting from short nucleotide stretches to the complete genome of an organism. Today, when one thinks of animal genomics and informatics, large number of databases are available to the open access of the researchers to explore the handy information pertaining to a species. So far, sequencing of chicken, fish and cow genomes have been completed and stored in the decentralized data repositories and many more are underway (Fadiel *et al.,* 2005). Farm animal genomics has multiple applications, such as in the quality assurance to confirm the percentage of a breed so as to get high-quality meat, to search for the disease-resistant genes and their selective breeding and so on (Appels, *et al.,* 2004 and Dove, 2005). Proteomics although has gained momentum in the post-genomic era; however, use of DNA based techniques in species identification outweigh the protein analysis techniques simply because the DNA is relatively thermostable and information coded in it is able to discriminate even the closely related species (Lenstra *et al.,* 2001).

The reasons why DNA is the molecule of choice for the purpose of species identification include (i) its stability allows the species identification in heated and processed products; (ii) DNA structure is conserved in all the tissues of an individual; (iii) higher discrimination of closely related species is possible as vast information is present in the

nucleotide algorithm consisting of A, T, G and C (Blackett and Keim, 1992; Buntjer *et al.*, 1995; Hunt *et al.*, 1997; Martinez and Malmhedenyman, 1998 and Wolf *et al.*, 1999).

The DNA based molecular tools could also identify rendered products, genetically modified food as well as breed and sex of the animals (Taylor and Woodgate, 1997; Brodmann and Moor, 2003 and Pfeiffer *et al.*, 2004). Candidate markers can identify particular species and also discriminate between pure and cross-bred animals (Aves *et al.*, 2002 and Sasazaki *et al.*, 2004). Detection of species by hybridization (crossing) in animals could also be accomplished using DNA based techniques such as PCR-RFLP, Sequencing, Satellite Fragment Length Polymorphism (SFLP or PCR-RFLP of satellite DNA) and the Micro Satellite Genotyping (Nijman *et al.*, 2003).

The DNA based techniques largely include PCR and its variants; PCR-RFLP, RAPD, DNA hybridization, PCR-sequencing, AP-PCR and PCR-SSCP. Further, quantitative analysis could be undertaken by using Real Time-PCR in order to assess the extent of adulteration. To accomplish this, both nuclear as well as the mitochondrial sequences could be targeted.

2.3.1 Targets for meat speciation

2.3.1.1 Nuclear targets for animal speciation

Animal species have been successfully identified using nuclear targets. However, the single or low copy number nature of nuclear targets may pose a limitation demanding some multi-copy targets. Hence, mitochondrial DNA sequences have been the best alternative over nuclear targets (Meyer *et al.*, 1994; Janssen *et al.*, 1998; Matsunaga *et al.*, 1998ab; Hopwood *et al.*, 1999 and Lockley and Bardsley, 2002b). PCR-based methods using multicopy nuclear sequences such as satellite DNA (Guoli *et al.*, 1999 and Calvo *et al.*, 2002a) and repetitive elements (Buntjer *et al.*, 1998; Buntjer and Lenstra, 1998; Calvo *et al.*, 2001b and Tajima *et al.*, 2002) have been used to differentiate animal species (Table 2.1). Most vertebrate animal species contain numerous nuclear actin genes with high sequence homology in comparable

exons, but have considerable variation in intron number and size, hence could be used for the animal speciation (Weber and Kabsch, 1994 and Lockley and Bardsley, 2002).

Short interspersed nuclear elements (SINE) have been targeted for the species identification (Lenstra *et al.,* 1993; Jobse *et al.,* 1995; Nijman *et al.,* 2002 and Walker *et al.,* 2003 and Mendoza-Romeo *et al.,* 2004). The SINEs could be used as phylogenetic markers in several eukaryotic taxa at different taxonomic levels (Nijman *et al.,* 2002). Contrary to most of the nuclear sequences that are present in a single copy, the advantage of targeting repeated sequences such as SINE are that these elements are repeated thousands of times in the genome and this helps in the species identification. Even when samples are processed and DNA is degraded, presence of multiple repeats/ copies helps in the PCR amplification. SINEs have also been used to generate unambiguous phylogenetic topologies relating to the eukaryotic taxa. The irreversible nature of SINE retro-position is supported by a large body of comparative genome data and is a fundamental assumption inherent in the value of this qualitative method of inference (Lum *et al.,* 2000). Various Interspersed Repeated Sequences/elements (IRSs) have been used to generate PCR-based multi-locus fingerprint profiles by amplifying the inter-element segments, using primers matching the elements themselves (Kostia *et al.,* 2000). The SINEs and Simple Sequence Repeats (SSRs) or microsatellites were used to establish the fingerprints and phylogenetic relationships of species (Kostia *et al.,* 2000). Development of PCR assays based on the short and long interspersed elements (SINE/ LINEs) has been the recent development for the animal species identification (Tajima *et al.,* 2002; Nicklas and Buel, 2003; Walker *et al.,* 2003 and Walker *et al.,* 2004).

2.3.1.2 Targeting mitochondrial DNA for animal speciation

Kocher *et al.* (1989) targeted the mt genes (cyt b gene) for the first time. The mitochondrial DNA is preferred over the nuclear DNA because of the following advantages:

- On an average, 1000 mitochondria per cell and 10 copies of mt DNA per mitochondria are present yielding $1x10^4$ copies of DNA per cell compared to just one copy number of the nuclear (genomic) DNA in a cell. Hence, in animal speciation, it improves the possibility of amplifying templates of suitable size in the samples containing even the fragmented DNA induced by heat denaturation (Long and David, 1980; Alberts *et al.,* 1994; Levin *et al.,* 1999 and Monttiel-Sosa *et al.,* 2000).
- Many DNA molecules within each mt make its DNA a naturally amplified source of genetic variation (Walker *et al.,* 2004)
- Designing of specific primers for PCR amplification is easy as the major region of mt DNA is conserved and most of the animal mt DNA/gene sequences are available in the public databases.
- Larger variability of mt DNA allows precise identification of species in samples derived from the mixed species as there is high rate of mutation in certain parts of mitochondrial DNA due to the poor corrective replication and lack of proof reading.
- Intra-species variability of mt DNA helps in the discrimination of even different breeds (evolutionary speciation) of animal species.

Specific PCR amplification of mitochondrial DNA targets has been the most appreciated and widely used method of animal species identification (Cann *et al.,* 1987; Kocher *et al.,* 1989; Meyer *et al.,* 1995; Unseld *et al.,* 1995; Matsunaga *et al.,* 1999ab; Wolf *et al.,* 1999; Partis *et al.,* 2000; Bellagamba *et al.,* 2001; Herman, 2001; Krcmar and Rencova, 2001; Sebastio, *et al.,* 2001; Parody *et al.,* 2002; Bellagamba *et al.,* 2003; Bottero *et al.,* 2003b and Dalmasso *et al.,* 2004).

The most common mt genes targeted for species identification include (Table 2.2) cytochrome b gene (Kocher *et al.,* 1989; Sullivan *et al.,* 1992; Holland *et al.,* 1993; Chikuni *et al.,* 1994a; Meyer *et al.,* 1995; Wilson *et al.,* 1995a; Zehner *et al.,* 1998; Bataille *et al.,* 1999; Matsunaga *et al.,* 1999ab; Wolf *et al.,* 1999; Dharnesh, 2006 and Halder, 2007), mt rRNA (5s, 12s, 18s etc) gene (Naito *et al.,* 1992; Matsunaga *et al.,* 1998ab; Rodriguez *et al.,*

2001 and Girish *et al.,* 2004, 2005) and D-loop (Foran *et al.,* 1988; Kocher *et al.,* 1989; Li and Graur, 1991; Wilson *et al.,* 1995ab; Lopez *et al.,* 1996 and Foran *et al.,* 1997). Additionally, recent studies have shown that there is a relationship between economic traits and mt DNA sequence variations in dairy and beef cattle and hence only these molecular markers can be used for the genetic selection (Schutz *et al.,* 1994; Boettcher *et al.,* 1996; Mannen *et al.,* 1998 and Mannen *et al.,* 2003).

2.3.1.2.1 Targeting mt D-loop for species identification

Replication in mammalian mt DNA proceeds from the initiation of heavy strand (H) synthesis at a specific origin (Crews *et al.,* 1979 and Gillum and Clayton, 1979), this results in the formation of displacement (D) loop with a newly synthesized H strand of ~680 bases known as 7s DNA (Anderson *et al.,* 1981). The light strand (L) synthesis also occurs at a specific origin but does not take place until the region is exposed by the H strand synthesis (Aloni and Attardi, 1971 and Murphy *et al.,* 1975). Mitochondrial transcription is a unique phenomenon due to the fact that both the strands seem to be completely transcribed from the promoters situated in the D-loop region (Aloni and Attardi, 1971 and Murphy *et al.,* 1975). These primary transcripts are further processed to yield 12S and 16S rRNAs, tRNAs and number of mRNAs which are uncapped and polyadenylated (Anderson *et al.,* 1981). The mt-D-loop does not code for a protein; however, it possess a very high rate of mutation and has been well characterized phylogenetically. Hence, in most of the species detection techniques, D-loop sequences have been targeted to amplify a portion of it (Foran *et al.,* 1988; Kocher *et al.,* 1989; Li and Graur, 1991; Wilson *et al.,* 1995 a & b; Lopez *et al.,* 1996 and Foran *et al.,* 1997). Also, in case of very old and highly degraded DNA specimens that no longer contain any nuclear DNA, usually mt sequences are targeted and D-loop is one among them (Anderson *et al.,* 1981; Kocher *et al.,* 1989; Holland *et al.,* 1993 and Kierstein *et al.,* 2004).

2.3.1.2.2 Targeting mt cytochrome b gene for species identification

The mt cytochrome b gene has been one of the markers useful in the forensic analysis (Kocher *et al.,* 1991; Chikuni *et al.,* 1994b; Forrest and Carnegie, 1994; Wilson *et al.,* 1995a; Lutz *et al.,* 1996; Abdulmawjood and Bülte, 2001, 2002). The cyt-b gene has been well characterized among different vertebrate animal species and the variation in its sequences among the species could be exploited for the purpose of species identification (Hatefi, 1985; Barallon, 1998; Irvin *et al.,* 1991; Zehner *et al.,* 1998; Parson *et al.,* 2000 and Verma and Singh, 2003). Since, sequencing could be costly for some laboratories; RFLP could be the better alternative after PCR amplification of mt cyt b gene (Meyer *et al.,* 1995 and Partis *et al.,* 2000).

Using these targets (either nuclear or mitochondrial), different DNA-based molecular techniques could be used to identify the animal species.

2.4 DNA-based techniques

The DNA based animal speciation techniques are the most preferred of all the techniques, since the DNA carries an organisms' total genetic information and is stably functioning as long as the animal is alive. The DNA isolated from any cell/tissue of an individual is identical irrespective of the organs or tissues. Further, the information available in the DNA is enormous as compared to the proteins due to the degeneracy of the genetic code. Hence, different DNA-based techniques used for animal species identification include DNA hybridization (Ebbehoj and Thomsen, 1991 a & b; Blackett and Keim, 1992; Hunt *et al.,* 1997 and Janssen *et al.,* 1998); PCR and its variants (Carr and Marshall, 1991; Meyer *et al.,* 1994 and Matsunaga *et al.,* 1999 a & b); PCR-RFLP (Meyer *et al.,* 1995 and Wolf *et al.,* 1999); RAPD-PCR (Welsh and McClelland, 1990; Williams *et al.,* 1990, Scheider *et al.,* 1997; Matsunaga *et al.,* 1999ab and Sebastio *et al.,* 2001); PCR-SSCP (Rehbein *et al.,* 1997,

1999; Asensio *et al.,* 2001 and Weder *et al.,* 2001), PCR-sequencing (Bartlett and Davidson, 1992), etc

2.4.1 DNA hybridization

DNA hybridization is a qualitative or semi-quantitative method of animal speciation which is based on the detection of species-specific DNA sequence (Ebbehoj and Thomsen, 1991a). Dot-blot technique was the first genetic approach for the species identification (Chikuni *et al.,* 1990 and Ebbehoj and Thomsen, 1991a). In DNA-DNA hybridization, there is a spontaneous association between two single complementary DNA strands leading to formation of a classical Watson and Crick duplex (Unseld *et al.,* 1995). Single stranded DNA is immobilized on to a nylon membrane known as the blot and the complementary DNA (either synthetic probe or another DNA) labeled with either an isotope (^{32}P), a fluorescent group (FITC), an antigen-hapten (digoxigenin), or an enzyme (alkaline phosphatase) is allowed to hybridize and the position of blot after hybridization is determined (Lenstra *et al.,* 2001).

In the early developments of animal species identification, species-specific probes were hybridized to the DNA extracted from the meat samples (Bauer *et al.,* 1987; Chikuni *et al.,* 1990; Wintero and Thomsen, 1990; Ebbehøj and Thomsen, 1991 a & b; Buntjer *et al.,* 1999 and Lenstra *et al.,* 2001). The species-specific probes (e.g. satellite repetitive DNA) enabled improved specificity and paved way for the detection of animal species even in the admixed meat with detection levels of <5% (Buntjer *et al.,* 1995; Hunt *et al.,* 1997; Janssen *et al.,* 1998; Buntjer *et al.,* 1999 and Lenstra and Buntjer, 1999).

A probe generated by PCR amplification of species specific satellite complementary DNA (cDNA) has also been used in hybridization (Table 2.3). Based on the conserved sequence contained in the actin multigene family, the genomic DNA was digested with *Bam*HI, electrophoresed and hybridized to α-actin cDNA probe to differentiate beef, pork,

lamb, horse, chicken and fish species (Janssen *et al.*, 1998). Although, it was difficult to differentiate the breeds within the species; however, analysts had attempted the combination of advantages of restriction digestion and the hybridization to differentiate the breeds (Matsunaga *et al*., 1998 a & b). However, the major disadvantages of DNA hybridization include time and labor, cross-reactivity of closely related species and interference in the processed samples. Further, in view of the complicated procedure and consequent to the unprecedented developments in molecular biology (biotechnology), the PCR has almost replaced the DNA hybridization for the purpose of species identification.

Table 2.3 DNA hybridization as a tool for animal species identification

	Species	**Assay**	**Target**	**Sensitivity**	**Reference**
1	Cattle	-	-	-	Wintero and Thomson, 1990
2	Pig	P^{32} labeled porcine probe	-	0.1% (raw); 0.5% (processed)	Ebbehoj and Thomsen, 1991a
3	Monkey, Human, Cattle, Goat and Sheep	Slot-blot hybridization	-	0.01%	Ebbehoj and Thomsen, 1991b
4	Rabbit, Sheep, Pig, Cattle and Goat	Species-specific probes	Satellite DNA	-	Hunt *et al.*, 1997
5	Chicken, Pig and Cattle	-	Satellite DNA	-	Buntjer *et al.*, 1999

2.4.2. PCR: A state-of-the-art technique for animal species identification

PCR provides a wide range of options to the analysts by virtue of its versatility and hence it is considered as state-of-the-art technique in animal species identification. It is a rapid means of making multiple copies of specific piece of DNA sequence *in vitro* and possess high level of selectivity as well as sensitivity and hence it is an ideal diagnostic analytical tool.

There are two PCR-based approaches where amplification of a genetic marker is carried out: 1. Mono-locus-specific primers for the amplification of a concrete DNA fragment and 2. Multi-locus amplification of a non-targeted DNA i.e., RAPD and AP-PCR (Welsch and McClelland, 1990; Williams *et al.*, 1990; Cushwa and Medrano, 1996 and Koh *et al.*,

1998). The difference between the two lies in their primer length (10 mer for RAPD-PCR and >18 mer for AP-PCR) and the stringency of amplification conditions (Saez *et al.*, 2004).

2.4.2.1 Techniques based on multi-locus primers

2.4.2.1.1 RAPD finger-printing

PCR-based fingerprinting techniques based on two different arbitrary DNA amplification approaches (namely RAPD and AP-PCR) have been employed for meat identification. Saez *et al.,* (2004) used a single step procedure for the identification of many meat species, where the method was rapid, simple and reproducible. Analysis of the resultant patterns was undertaken using suitable softwares such as calculation of similarities of the band profile using algorithm and statistical programmes.

The RAPD uses non-specific primers based on short oligonucleotide sequences to amplify many unidentified sequences simultaneously leading to a reproducible pattern of PCR products upon gel electrophoresis and the unique species-specific fingerprints generated help in the species discrimination (Lee and Chang, 1994; Rao *et al.*, 1996; Koh *et al.*, 1998; Martinez and Malhedenyman, 1998; Ganai *et al*., 2000; Calvo *et al.*, 2001a and Saez *et al.*, 2004). Advantages of RAPD fingerprinting as a diagnostic tool are: (a) It gives species specific pattern and (b) it does not require any previous knowledge of the DNA sequence of a species. But the major disadvantage of RAPD lies in its inability to detect mixed species (meat) and it also lacks quantification of the extent of adulteration.

Although, RAPD-PCR is a rapid and discriminative procedure but the intra-species polymorphism and PCR conditions sometime interfere with detection. Higher number of polymorphisms in-between and lesser within a species aid in the generation of RAPD fingerprints. However, always such profile may not be sufficient to differentiate all the species any time because of weak reproducibility of such profiles (Koh *et al.,* 1998).

Cleavage of Amplified Polymorphic Sites (CAPS) or RAPD could be used for mass screening of samples that offer a broader range but such techniques usually need intact DNA with preserved integrity and hence they could be less suitable for the analysis of processed food samples that require lot of difficult standardizations (Lopez-Andreo *et al.,* 2005).

2.4.2.1.2. Use of micro-satellite markers

Individual multi-locus genotype analysis based on the micro satellite markers had been used for the species identification even up to breeds (Ciampolimi *et al.,* 2000). Micro satellite markers located on different chromosomes could be amplified and analyzed to aid in traceability of the animal species (Arena *et al.,* 2002). A novel micro satellite (STR) marker has been used for the forensic identification of big cats in India (Singh *et al.,* 2004). Similarly, beef was also identified in meat mixtures based on micro-satellite markers (Shackell *et al.,* 2005).

2.4.2.1.3 Amplified Fragment Length Polymorphism (AFLP)

The AFLP has been used to discriminate between cattle breeds, where about 500 primer combinations yielded selected markers that were converted into a single nucleotide marker for the high throughput genotyping of the cattle (Sasazaki *et al.,* 2004).

2.4.2.2 Techniques based on mono-locus primers

Most of the recent techniques use amplification of the target DNA either by the use of species-specific primers or the use of universal primers (Fairbrother *et al.,* 1998; Matsunaga *et al.,* 1999; Lockery and Bardsley, 2002). This approach requires prior knowledge of the nucleotide sequences to be used as a target, design of the mono-specific primers and its validation (Meyer *et al.,* 1994; Lockery and Bardsley, 2002). Further, while working with universal primers, intra-specific polymorphisms need to be taken care of by using either the product sequencing, hybridization, RFLP or SSCP (Wolf *et al.,* 1999; Martinez and Danielsdottir, 2000; Bellagamba *et al.,* 2001 and Myeres *et al.,* 2003). However, for the

initial analysis, beginning could be accomplished by the non-targeted multi-locus amplification such as RARD-PCR or any other alternative (Koh *et al.*, 1998; Martinez and Malmhedenyman, 1998 and Calvo *et al.*, 2001a).

2.4.2.2.1 Species-Specific PCR

Species specific DNA in femto grams (fg) and pico grams (pg) could be detected in both processed and unprocessed samples using targeted amplification of rRNA genes (12S, 16S, 18S), actin-multigene families (which are highly conserved in all the eukaryotes), satellite DNA, cytochrome-b gene, cytochrome oxidase II, growth hormone gene, melanocortnin gene, mt D-loop, myofibrillar components and Satellite I DNA using PCR (Pallard and Cooper, 1986). Very old samples (as old as 120-135 million years) could also be amplified in PCR (Cano *et al.*, 1993).

Some highly conserved regions on the mitochondrial genes such as cytochrome b have been most widely used compared to others (Table 2.2) for the species identification where pair of specific primers amplifies a specific region in PCR (Kocher *et al.*, 1989; Meyer *et al*, 1995 and Wolf *et al.*, 1999). Although, both genomic and mt targets are used (Table 2.4), mt sequences are preferred since these possess high rate of mutation allowing species identification and also help in the differentiation of most closely related species (Wolf *et al.*, 2000).

Table 2.4 Application of conventional PCR for the animal species identification

	Species	Target	Sensitivity	Reference
1	Pig	SINE	0.005% in beef; 1% in duck	Calvo *et al.*, 2001b
2	Pig	GH gene	<2%	Wolf and Luthy, 2001
3	Pig	SINE	1%	Calvo *et al.*, 2002a
4	Chicken, Turkey	α cardiac actin	1%	Lockley and Bardsley, 2002
5	Many species	genomic (β-actin; TP53), mt (cyt-b; D-loop; 28s rRNA)	-	Bellis *et al.*, 2003
6	Goose, Duck	α-actin gene	1%	Rodriguez *et al.*, 2003
7	Cow, Sheep, Goat	mt-12S rRNA gene	0.1%	Lopez-Calleja *et al.*, 2004
8	Partridges	Ribosomal- ITS	1 ng	Tejedor *et al.*, 2006

2.4.2.2.2 Multiplex PCR

Multiplex PCR offers a short cut to many individual PCRs as many targets are simultaneously amplified. Keeping a primer common, species specific forward/reverse primers could be used for the simultaneous detection of many species (Cespedes *et al.,* 1999a & b). Multiplex PCR saves time, labor and it is a good tool for the mass screening of samples that might be confirmed later by species-specific PCR. Goose and duck meat were differentiated using a multiplex PCR with a common forward and species-specific reverse primers to nuclear repeated target (5S rDNA gene) in order to detect the fraudulent substitution of a duck liver for the more costly goose liver (Rodriguez *et al.,* 2001). Six species were identified simultaneously by targeting cyt-b gene in a multiplex-PCR (Matsunaga *et al.,* 1999). Cyt-b and mt-D-loop targets were used to differentiate human and animals using a multiplex PCR where appearance of two bands was indicative of human and a single band in the animals (Bataille *et al.,* 1999).

2.4.2.2.3 Nested and hemi-nested PCR

If the DNA samples are suspected to be degraded (processed foods), it is wise to go for a two stage PCR process: initial amplification using low stringency primer pair and the second one with a nested PCR using specific primers so as to yield a unique band specific to a species (Hopwood *et al.,* 1999). Based on the polymorphisms in the mitochondrial 16S rRNA gene, blackbuck, goral, nilgai, hog deer, chital, sambar and thamin deer were identified using nested PCR. Firstly, a ~550 bp region of the 16S rRNA gene was amplified by PCR using template DNA and universal primers and in the second stage, a species-specific internal region of the gene was amplified using PCR amplified product of the first species-specific primers. The amplicon generated after two consecutive amplifications was highly unique to the target species (Guha and Kashyap, 2005).

2.4.2.2.4 Restriction Fragment Length Polymorphism (RFLP)

In PCR-RFLP, the conserved target gene is amplified and the resultant PCR product is digested with specific restriction endonuclease enzyme (s) to get a restriction pattern (fingerprint). These restriction enzymes splice the DNA at specific sites to yield species-specific pattern (Meyer *et al.,* 1995 and Murray *et al.,* 1995). PCR-RFLP is a rapid and versatile tool but often requires technical competence in interpretation of results (Lockley and Bardsley, 2000 a). The mutated nucleotides are recognized by the restriction enzymes leading to a specific recognition of the sequence. The mitochondria accumulate 10 times more mutations per unit time compared to the nuclear sequences. Hence, mt DNA has been the most targeted sequence. Also, PCR-RFLP has been widely used for species identification especially targeting the mt cyt-b gene (Meyer *et al.,* 1995 and Meyer and Candrian, 1996).

Different restriction enzymes (*Afi*III, *Alu*I, *Apa*I, *Ava*II, *Bam*HI, *Dra*I *Eco*RI, *Hinf*I, *Nco*I, *Nsp*I, *Pst*I, *Rsa*I, *Sfu*I, *Sty*I and *Taq*I) could be used depending on the enzyme recognition and splicing sites. The PCR-RFLP has been one of the most commonly used techniques for the differentiation of meat species (Bossier, 1999; Wolf *et al.,* 1999; Matsunaga *et al.,* 1999ab and Wolf *et al.,* 2000).

Using PCR-RFLP, it is possible to identify even a single point mutation on restriction site as the restriction pattern would change after mutation. Many qualitative PCR-RFLP methods have been popularly used by scientists for the identification of chicken (Hopwood *et al.,* 1999 and Matsunaga *et al.,* 1999 a & b), goat (Chikuni *et al.,* 1994 b and Matsunaga *et al.,* 1999 a 7 b), beef (Matsunaga *et al.,* 1999 a & b), sheep (Chikuni *et al.,* 1994 a and Matsunaga *et al.,* 1999), pig (Meyer *et al.,* 1994; Matsunaga *et al.,* 1999 and Montiel-sosa *et al.,* 2000), horse (Matsunaga *et al.,* 1999) and fish (Cespedes *et al.,* 1999 a) meats (Table 2.5).

PCR-RFLP has been used in the identification of food animals even in the samples containing degraded DNA with apomorphic sites (Meyer *et al.,* 1995, Wilson *et al.,* 1995a; Ram *et al.,* 1996; Carney *et al.,* 1997; Plath *et al.,* 1997; Rigaa *et al.,* 1997; Yoshizaki *et al.,* 1997; Carrera *et al.,* 1998, 1999; Cespedes *et al.,* 1999a; Abdulmawjood and Bülte, 2001 and Pfeiffer *et al.,* 2004). The major disadvantage of PCR-RFLP is its inability to identify a species in the mixed samples and in such cases species-specific PCR overweighs the RFLP (Cespedes *et al.,* 1999 a).

Table 2.5 Use of PCR-RFLP for the identification of animal species

	Species	Target	Enzymes	Detection	Reference
1	Chicken; turkey	Alpha-cardiac actin gene	*Rsa*I; *Hae*III	<1%	Hopwood *et al.,* 1999
2	Mammals	mt $tRNA^{Glu}$/Cyt-b	-	-	Wolf *et al.,* 1999
3	Pork	mt D-loop	*Ava*II	5%	Montiel-Sosa *et al.,* 2000
4	Many species	mt cyt-b gene	*Hae*III; *Hinf*I	1%	Partis *et al.,* 2000
5	Ostrich	mt cyt-b gene	*Hae*III; *Hinf*I; *Rsa*I; *Tru*9I	-	Abdulmawjood and Bulte, 2002
6	Bovine, pig, sheep, chicken	ATPase	*Hph*I; *Mnl*I; *Ssp*I; *Hind*III	1%	Cheng *et al.,* 2003
7	Cattle, sheep, Goat, deer	mt cyt b gene	*TSP*509	-	Pfeiffer *et al.,* 2004
8	Cattle, buffalo, sheep, goat	mt rRNA (12s) gene	*Alu*I; *Hha*I; *Apo*I; *Bsp*TI	-	Girish *et al.,* 2005

2.4.2.2.5 Sequence analysis of PCR products

Sequencing of the PCR products and their sequence analysis could discriminate the animal species (Bartlett and Davidson, 1992; Sullivan *et al.,* 1992; Palumbi and Cipriano, 1998; Burger *et al.,* 1999 and Brodmann *et al.,* 2001). The nucleotide sequence similarity and the divergence between the species aid in differentiation. Mitochondrial 12S rRNA gene sequence analysis has been used to differentiate cattle, buffalo, sheep, goat, mithun, low land anoa, domestic yak and pig (Girish *et al.,* 2004). However, there are certain limitations of this technique for its exclusive use for animal species identification, that include-

- Sequencing can not be used for the differentiation of admixed (adulterated) samples, as these contain amplicons of different species and lead to ambiguous interpretation.
- One-strand partial DNA sequencing could not be considered definitive in the court of law (Colombo *et al.,* 2004).
- Sequencing is somewhat costly and sufficient length of species' sequence information should be existent in the sequence database (Quinteiro *et al.,* 1998).
- There should be minimum intra-species variability so as to avoid mis-identification.
- Heteroplasmy (different sequences in the same individual) should be absent and the diagnostic positions i.e., exclusive nucleotide pattern at certain positions should be taken into account (Quinteiro *et al.,* 1998). If there is overlapping distance values, the intra-species and inter-species variability can not be explored using genetic distance based analysis and in such cases sequencing can not be considered as a technique of choice for the animal species differentiation.
- Legally, combination of two molecular techniques such as a protein-based and a PCR-based technique completes the analysis, as possibility of SNP (single nucleotide polymorphism) between the closely related species could interfere the conclusive decision.

The PCR product sequencing of amplicons obtained with a pair of universal primers of cyt-b gene has been successfully used for the purpose of species identification, where the results were counterchecked using IEF (Colombo e*t al.,* 2004). Apart from cyt-b gene, mt 12S rRNA gene was also used for this purpose (Prakash *et al.,* 2000). Further, sequencing of PCR product followed by computation using softwares available in websites has been used for the identification of an unknown species (Brodmann *et al.,* 2001).

For the identification of samples, the determined sequence is put in the distance matrix analysis and a tree is constructed. The putative sample is then clustered with the

sequences of reference species to which the sample is most phylogenetically related (Quinteiro *et al.,* 1998). After determining the sequence of a specific target, phylogenetic trees could be constructed and the species could be traced out. For instance, determination of the complete sequences of mt cyt b (1,137 bases) and 12S rRNA (961 bases) genes from the woolly mammoth (*Mammuthus primigenius*), African (*Loxodonta illiams*) and Asian (*Elephas maximus*) elephants; phylogenetic relationships were examined and relatedness was explored for their identification (Noro *et al.,* 1998).

PCR and sequencing is worth for animal species identification since very small amount of sample is required and use of universal primers help in the differentiation of wide range of species (Colombo *et al.,* 2004). Further, PCR product sequencing has high level of precision and accuracy that merits its use in animal species identification.

Also, restriction imposed by mutations in the use of PCR-RFLP could be solved by sequencing (Bartlett and Davidson, 1991, 1992 and Ram *et al.,* 1996). However, sequencing of PCR product is time consuming, costly and may not be accessible to all. But, enormous data have been put in the public databases to facilitate the application of sequencing as one of the adjuncts for the animal species identification.

Forensically Informative Nucleotide Sequencing (FINS) - For meat species identification, a forensically informative fragment of a particular gene is made use of to solve the legal problems. The conserved region of a gene specific to a species is amplified and the PCR product is cloned, sequenced and the sequence is analyzed (Bartlett and Davison, 1992 and Forrest and Carnegie, 1994). A specific PCR assay was developed for the identification of tiger in the meat, feces and dried skin samples in order to curb the illegal trade in wildlife (Wan and Fang, 2003). A species-specific PCR would be rather appreciable better if the samples are sub-optimal in the forensic analysis over the RFLP.

2.5 Quantitative meat speciation

Quantification of extent of meat adulteration is essential especially in meat products such as sausage and other products. Earlier, quantitative techniques involved DNA extraction, hydrolysis with trichloroacetic acid, reaction with diphenylamine and spectrophotometric measurement of the resultant complex at 600 nm (Burton, 1956 and Buntjer *et al.,* 1999). Later on, DNA hybridization was used for the quantitative analysis where the size of DNA was determined by agarose gel electrophoresis, immobilized on to nylon membranes and hybridization was carried out using labeled probes made from known labeled DNA (^{32}P); then the signal intensity of filter bound DNA probe was measured using laser densitometry/ autoradiography (Ebbehoj and Thomsen, 1991a).

Apart from densitometry (Calvo *et al.,* 2002 b), the PCR-based techniques are available today that can be used for qualitative, semi-quantitative and quantitative analysis (Laube *et al.,* 2001). PCR-based quantitative techniques used for animal speciation include quantitative-competitive PCR (Wolf and Luthy, 2001 and Frezza *et al.,* 2003); and the real time PCR (Brodmann and Moor, 2003; Sawyer *et al.,* 2003; Walker *et al.,* 2003; Rodriguez *et al.,* 2004a and Lopez-Andreo *et al.,* 2005). In quantitative-PCR, single copy genes (such as glyceraldehyde-3-phosphate dehydrogenase -GAPDH) were preferred over the multi copy mt genes (Woolfe and Primrose, 2004).

2.5.1 Quantitative Competitive PCR (QC- PCR)

Conventional PCR is dependent on the end point detection i.e., concentration of the target. Hence, it fails to accurately measure the amount of target DNA in the original sample. This happens because the PCR product does not increase in a linear fashion as (Woolfe and Primrose, 2004). Hence, the quantitative assays should measure the amount of initial target DNA. The QC-PCR relies on the amplification of an internal standard at the same time the target DNA (Larrick 1997 and Wiseman, 2002). This internal standard act as competitor to

the target sequence during the reaction and direct comparison of the products generated from the two help in the quantification. QC-PCR on porcine DNA using porcine growth hormone gene and a competitor differing 20 bp in length from the porcine target was constructed and used (Wolf and Luthy, 2001).

2.5.2 Real-Time PCR

The conventional PCR developed for various species are purely qualitative (Tanabe *et al.*, 2007b) and there has always been a search for a quantitative assay. Advent of Real-Time PCR has revolutionized the field of molecular biology and could be employed for the animal species identification as well as quantification (Holland *et al.*, 1991; Higuchi *et al.*, 1993; Heid *et al.*, 1996 and Wittwer *et al.*, 1997).

The real time PCR used for meat speciation is available with numerous chemistries (Table 2.6). In TaqMan™ chemistry, a fluorogenic probe binds to the target sequence between the flanking primers which is labeled on either side with a reporter and a quencher molecule. As polymerization begins the 5'-3' exonuclease activity of *Taq*-DNA polymerase cleave the hybridized probe releasing the 5'-reporter that emits fluorescence and is measured in real time. Hence, the possible post-PCR sample handling and chances of contamination are not encountered in real the time PCR (Rodriguez *et al.*, 2005).

The advantage of TaqMan™ probes is that MGB-oligodeoxynucleotide conjugate shows high affinity to the target allowing short (13-16mer) nucleotides to be used as probes and species-specific targeting discriminates the closely related species. Selective primers and fluorogenic MGB probes to inter-species-specific targets labeled with 3' mismatches have been used for the species discrimination (Lockley and Bardsley, 2002; Lopez-Andreo *et al.*, 2005).

Table 2.6 Use of real time PCR for animal species identification

	Species	Target	Chemistry	LOD	Reference
1	Cattle	GH gene	TaqMan™	0.01% (0.02 ng)	Brodmann and Moor, 2003
2	Cattle	16S rRNA	SYBR Green I, Scorpion probes	0.1%	Sawyer *et al.*, 2003
3	Pig, Chicken, Ruminants	SINE / LINE	SYBR Green I	0.01-1 pg	Walker *et al.*, 2003
4	Cattle, Pig, Sheep, Chicken, Turkey	Cyt-b gene	TaqMan™	0.1-0.5%	Dooley *et al.*, 2004
5	Ruminants	SINE Bov-A2.	Semiquantitative	10 fg (10%)	Mendoza-Romero *et al.*, 2004,
6	Aves, Equine, Canine, Feline, Rodents	SINE / LINE	SYBR Green I	0.1 ng- 0.1 pg	Walker *et al.*, 2004
7	Bovine, Porcine, Sheep, Chicken, Turkey, Ostrich	mt $tRNA^{Glu}$; ND5; Cyt b	TaqMan MGB	0.03-0.8 pg (1%- >5%)	López-Andreo *et al.*, 2005
8	Horse, Donkey	Cyt-b gene	-	1 and 25 pg	Booth *et al.*, 2005
8	Pig	12S rRNA	TaqMan™	0.5-5%	Rodriguez *et al.*, 2005
9	Ruminants	$tRNA^{Lys}$/ ATP8	TaqMan probe (FAM–TAMRA)	0.1%.	Fumière *et al.*, 2006
10	Cattle, Wallaroo, Pig, Horse	-	SYBR Green I	0.04-0.4pg (1-5%)	López-Andreo *et al.*, 2006
11	Bovine	Cyt-b gene	TaqMan™ (FAM-labeled)	35pg	Zhang *et al.*, 2007
12	Pig, Chicken, Cattle, Sheep, Horse	Cyt b gene	TaqMan™ MGB probes	100 fg	Tanabe *et al.*, 2007a

In this direction, to confer the specificity to the real-time assay, two complementary strategies have been used i.e., placing the 3' end of the primer at a point of sequence heterogeneity and by truncating the primers at the 5' end to lower the calculated melting temperature. Employing these strategies, a selective and specific assay on a conserved region of mt cyt-b gene was used for the unambiguous detection of species (Hird *et al.*, 2004).

Using real time PCR, a linear plot (calibration curve) of the known DNA/ concentration (x-axis) versus the Ct values (y-axis) gives a linear equation [$y = mx + c$, where y is Ct, x is log DNA concentration, m is slope and c is constant] with the acceptable

regression coefficient (r^2> 0.99) would be obtained that help in the quantification of unknown DNA (Rodriguez *et al.,* 2005). Thus, the Real Time PCR assay employed for the meat speciation helps in the analysis of the reaction kinetics in real time allowing the quantification of target DNA. It eliminates procedures such as post PCR processing since both amplification and detection are undertaken in a single tube. Further, avoidance of ethidium bromide use and exposure to the UV-light eliminates hazard to the handlers. Large scale screening of samples in less time could also be done. The Ct is a more reliable measure of starting DNA than the end point detection where the later just measures the accumulated DNA in the conventional PCR (Lahiff *et al.,* 2002). Hence, high processing speed, automation, specificity, sensitivity and reliability make the real time PCR a tool of choice to the analysts for the purpose of species identification (Weller *et al.,* 2000 and Rodriguez *et al.,* 2005).

The cost involved in the TaqMan™ chemistry could be reduced if SYBR Green I based chemistries are used, where a fluorescent dye is used in place of oligonucleotide probes (Sawyer *et al.,* 2003 and Walker *et al.,* 2003, 2004). Further, the results obtained from the SYBR Green I uniplex and duplex reactions with single-species DNA are comparable to TaqMan™ probes (López-Andreo *et al.,* 2006).

Further, in real-time PCR, both single and the multicopy targets could be used for the species identification and quantification (Laube *et al.,* 2003 and Rodriguez *et al.,* 2004a). Also, multi species-specific probes for the quantitative speciation have been reported (Rodriguez *et al.,* 2004a). In the real-time PCR, the specificity is due to the primers especially while using TaqMan™ chemistry, the MGB probes ensure the amplification genuinely and reduce the uncertainty associated with the background signals (Lopez-Andreo *et al.,* 2005).

However, amplicon size has got a direct bearing on the sensitivity (relation between amplification rates and amplicon size) in real-time analysis. The amplicon size should be as small as possible (Hird *et al.*, 2006). The reproducibility of the real-time-PCR between the laboratories and repeatability within a laboratory are high and hence the confidence level of real time PCR in any laboratory is higher than other techniques (Abdulmawjood *et al.*, 2006).

The application of real time PCR for the quantitative analysis include, amplification and detection in real time, earliest detection of PCR product, high efficiency, multiplex capability using different fluorescence spectra, freedom from the post-PCR sample handling and opportunity for the 'normalization' and quantification.

Real time PCR has the ability to speciate common meat animals including the poultry and fish in mixtures. Apart from quantitative capabilities, the real time PCR possesses better sensitivity and rapidity (Tanabe *et al.*, 2007 a). However, complex analysis, difficult standardizations, and costly equipment may sometimes hinder its selection by some analysts.

2.6 Animal speciation to control BSE and other zoonoses

After the report of the BSE in 1986 in U.K., there has been a ban on the use of ruminant or mammalian derived material in the animal feed (Wells *et al.*, 1987; Decision 96/449/ European Commission 1996; Will, 1999; Cheng *et al.*, 2003). Further, consumer and farmers' confidence needs to be restored in the event of TSEs, FMD and zoonoses that have health and trade concerns (LMV 1995; TSV 1995; Woodhouse *et al.*, 2001; Kurzenhauser, 2001; Woodhouse *et al.*, 2001). To control the spread of BSE, identification of ruminant derived material is essential in the feed and PCR-based methods have been described for the detection of the species in meat and MBM (Table 2.7). Most of these methods are based on amplification of mt DNA (Cheng *et al*., 2003).

Table 2.7 PCR-based speciation to detect ruminant derived products in animal feed

	Species	Target	Assay	Sensitivity	Reference
1	Beef	$tRNA^{Lys}$-ATPase 8	PCR-RFLP (*Dpn*II & *Ssp*I)	<0.125%	Tartaglia *et al.*, 1998
2	Ruminant	Bov ATPase 6, 8	PCR	-	Wang *et al.*, 2000; Krcmar and Rencova, 2001
3	Ruminant	Cyt-b	PCR-RFLP	-	Bellagamba *et al.*, 2001
4	Ruminants	Bov ATPase 8	PCR	-	Colgan *et al.*, 2001
5	Ruminant	$tRNA^{Glu}$, Cyt-b	PCR	-	Kingombe *et al.*, 2001
6	Ruminant	ATPase 8	PCR	-	Lahiff *et al.*, 2001
7	Beef	1709 satellite	PCR	0.005%-1%	Calvo *et al.*, 2002b
8	Ruminant	Bov ATPase 8	PCR (online 5' Nuclease)	-	Lahiff *et al.*, 2002
9	Ruminant	SINE (bov-B, PRE-1, CR-1)	PCR	-	Tajima *et al.*, 2002
10	Ruminant	12s rRNA gene	PCR	-	Bellagamba *et al.*, 2003
11	Mammals	Bov GH	Real time PCR (TaqMan™)	0.02 ng (0.01%)	Brodmann and Moor, 2003
12	Ruminant	Bov ATPase 6 & 8	PCR (competitive)	-	Frezza *et al.*, 2003
13	MBM	SINE	Real time PCR	10 fg	Mendoza-Romero *et al.*, 2003
14	Ruminant	SINE (1.711B, PRE-1, CR1, Bov-tA)	Real time PCR (on-line SYBR fluorescence)	-	Walker *et al.*, 2003
15	Ruminant	SINE (bov-A2)	PCR (online 5' nuclease)	10 fg	Mendoza-Romero *et al.*, 2004
16	MBM-Bovine, Sheep	SINE (bov-B)	Real time PCR (SYBR Green)	0.1%	Aarts *et al.*, 2006
17	CNS tissue in meat	GFAP- mRNA	Real time PCR	0.1%-0.2%	Abdulmawjood *et al.*, 2006
18	Meat and MBM in feed	mt $tRNA^{Lys}$/ ATP8	Real time PCR (TaqMan™, FAM–TAMRA)	0.1%.	Fumière *et al.*, 2006

2.7 Application of animal speciation techniques in wild-life forensics

In wildlife forensics, PCR amplification using a universal primer and sequencing has been employed by several workers (Kocher *et al.*, 1989; Bartlett and Davison, 1992; Parson *et al.*, 2000; Verma *et al.*, 2003; Verma and Singh, 2003 and Gupta *et al.*, 2005), as shown in Table 2.8.

Tracking and seizure of the largest ivory trade and its ban in the year 1989 was accomplished using a "Voronoi tessellation method", where genetic similarities across the tusks inferred the origin of multiple samples (Wasser *et al.*, 2007). Similarly, phylogenetic evolution of antler deer of mt DNA (rRNA & tRNA genes) was used for the deer species identification (Miyamoto *et al.*, 1990). Species-specific PCR was also used to identify tiger meat thereby solving a forensic case using a single hair (Wan and Fang 2003). Using PCR amplification and sequencing of mt 12S rRNA gene fragment, the Indian muntjac (*Muntiacus muntjak*) was identified (Shukla *et al.*, 2001).

Most of the forensic laboratories receive partially or completely degraded samples, hence the methodologies used should be sufficient enough to draw the conclusive results oftenly as there will be no nuclear DNA and in such cases targeting the mitochondrial DNA is advocated (Bataille *et al.*, 1999; Wan and Fang, 2003). Further, urgent confirmation required by the legal authorities could be met by employing species-specific PCR or PCR-RFLP since PCR-sequencing take considerable time (Bottero *et al.*, 2002).

Table 2.8 PCR based techniques for solving problems of wildlife forensics.

	Species	Target	Assay	Reference
1	Deer	rRNA & tRNA genes	Phylogenetic analysis of mt DNA	Miyamoto *et al*., 1990
2.	Big cats	D-loop, cyt-b gene	PCR-sequencing	Shankaranarayanan and Singh, 1998
3	Felines (Lion, Tiger)	Micro (Fca 45, 77, 126), D-loop	PCR	Shankaranarayanan and Singh, 1998
4	Bovine, Tiger	12S rRNA gene	PCR-sequencing	Prakash *et al*., 2000
5	Indian muntjak	12S rRNA gene	PCR-sequencing	Shukla *et al.*, 2001
6	Indian peafowl (Peacock)	Cyt b gene	PCR	Gupta *et al.*, 2005
7	Fish	mt DNA	Real time qPCR (SYBR Green)	Deagle and Tollit, 2007

2.8 Other less commonly used techniques for animal speciation

Directly Amplified Length Polymorphisms (DALP) uses AP-PCR to produce genomic fingerprints and enable sequencing of the DNA polymorphisms in virtually any species. Many species could be differentiated using DALP. Here, prior knowledge of the target or its cloning is not required; however, at times the results are not reproducible (Desmais *et al.*, 1998).

Single Strand Conformation Polymorphisms (SSCP) is a secondary structure analysis tool. Wild, domesticated and hybrid partridges were identified by using PCR-SSCP (Tejedor *et al.*, 2006).

Simple Sequence Repeat Polymorphisms (SSRP) is used to distinguish varieties using (>40) primer sets. The technique hold high level of discrimination, however, information of the DNA sequence is required, and there is difficulty in obtaining and maintaining reference materials and it also requires complex instrumentation.

In **Inter-Retrotransposon Amplified Polymorphisms (IRAP),** the retrotransposons are analyzed for speciation located in the genome and that show genetic differences when

amplified using specific primers yielding reproducible banding patterns that helps to indicate geographical origin of the species and in their authentication.

New generation "***DNA invader assays"*** aim at detection without amplification using a generic enzymatic method and Fluorescence Resonance Energy Transfer (FRET) for the signal amplification and lessen the differences in the efficiency demonstrated by the PCR. However, the sensitivity is a major constraint.

Although number of highly specific DNA based techniques have been developed for the purpose of species identification, however, most of these are just qualitative at present as absolute quantification is not possible. Certain animal product matrices such as gelatin powder and meat broth are processed under such conditions where the total DNA gets degraded or leached out into the liquid wash; even if it remains we need to have very fine tuned technology to extract DNA from such samples. In such cases applicability of the PCR in particular becomes questionable (Brodmann and Moor, 2003). Hence, there has been a search for the techniques that could detect a species, discriminate from the most closely related ones and accurately quantify the extent of adulteration.

Table 2.1 Nuclear targets employed for species identification

	Nuclear target sequence	Species	Reference
1	Alpha DNA Satellite	Primates	Waye *et al.*, 1989
2	Satellite I	Sheep, Goat	Chikuni *et al.*, 1994a
3	Satellite DNA- Alu-repeats	Primates	Novick *et al.*, 1995
4	Satellite DNA- Short-tandem repeats; variable number repeats	Human	Crouse and Schumm, 1995; Latorra and Schanfield, 1996; Sparkes, 1996;
5	1.709 satellite DNA	Beef	Guoli *et al.*, 1999
6	1709 satellite	Beef in feed	Calvo *et al.*, 2002b
7	Cyt oxidase II, satellite IV, satellite 1.711b	Cattle	Verkaar *et al.*, 2002
8	Actin multigene family	Beef, Pork, Lamb, Horse, Chicken, Fish	Fairbrother *et al.*, 1998
9	Actin multigene family	Chicken	Hopwood *et al.*, 1999
10	Alpha-cardiac actin gene	Chicken, Turkey	Lockley and Bardsley, 2002
11	Beta-actin; TP53 gene	Human, Pig, Chicken, Cow	Bellis *et al.*, 2003
12	Alpha actin gene	Goose, Duck	Rodriguez *et al.*, 2003
13	GH gene	Pork	Wolf and Luthy, 2001
14	Bov GH gene	Bovine, Mammals	Brodmann and Moor, 2003
15	5s rRNA gene	Fish	Cespedes *et al.*, 1999b; Carrera *et al.*, 2000
16	Bovine ATPase subunits 6 and 8	Bovine, Ruminants	Wang *et al.*, 2000; Colgan *et al.*, 2001; Lahiff *et al.*, 2001 Krcmar and Rencova, 2001; Lahiff *et al.*, 2002; Frezza *et al.*, 2003
17	Short Interspersed Nuclear Elements (SINE)	Vertebrates	Silja-Kostia *et al.*, 2000; Calvo *et al.*, 2001b, 2002a; Nijman *et al.*, 2002
18	SINE-bovine (bov-B), porcine (PRE-1), Avian (CR1)	Ruminants, Porcine, Chicken	Tajima *et al.*, 2002
19	SINE-bovine (satellite 1.71B), porcine (PRE-1), Avian (CR1), Ruminant (Bov-tA)	Cow, Pig, Chicken, Ruminants	Walker *et al.*, 2003; Walker *et al.*, 2004
20	SINE Ruminant (Bov-tA2)	Ruminants	Mendoza-Romero *et al.*, 2004
21	SINE Ruminants (Bov-B)	Bovine, Sheep	Aarts *et al.*, 2006

Table 2.2 Mitochondrial targets used for animal species identification

	mt target	Species	Reference
1	Cyt-b gene	Fish	Rehbein *et al.*, 1997; Carrera *et al.*, 1998; Cespedes *et al.*, 1998; Quinteiro *et al.*, 1998
		Deer	Matsunaga *et al.*, 1998a
		Cattle, Pig, Chicken, Sheep, Goat, Horse	Matsunaga *et al.*, 1999a & b
		Pig, Chicken, Horse, Cattle	Bataille *et al.*, 1999
		Ostrich, Emu	Colombo *et al.*, 2000
		Many species	Partis *et al.*, 2000
		Ruminant	Bellagamba *et al.*, 2001
		Goose	Colombo *et al.*, 2002
		Chicken, Turkey	Hird *et al.*, 2003
		Buffalo	Rajapaksha *et al.*, 2003
		Animals, Human	Bellis *et al.*, 2003
		Tiger	Wan and Fang, 2003
		Cattle, Sheep, Pig, Chicken, Turkey, Mammal, Poultry	Dooley *et al.*, 2004
		Cattle, Sheep, Goat, Deer	Pfeiffer *et al.*, 2004
		Peafowl	Gupta *et al.*, 2005
		Cow, Goat, Sheep	Maskova and Paulickova, 2006.
		Pig, Chicken, Cattle, Sheep, Horse	Tanabe *et al.*, 2007a & b
		Cow, Chicken	Sawyer *et al.*, 2003
2	16S rRNA gene	Animal species	Bellis *et al.*, 2003
		Pecora	Guha and Kashyap, 2005
		Cattle, Pig	Fumiere *et al.*, 2006
3	18S rRNA, cyt b, $tRNA^{Glu}$, ND5 genes	Pork	Montiel-Sosa *et al.*, 2000
4	28S rRNA gene	Cattle, Pork, Sheep, Chicken, Turkey, Ostrich	Lopez-Andreo *et al.*, 2005
5	12S rRNA gene	Fish	Asensio *et al.*, 2000
		Muntjak	Shukla *et al.*, 2001
		Ruminant, Porcine, Poultry	Bellagamba *et al.*, 2003
		Cattle, Buffalo, Sheep, Goat	Girish *et al.*, 2004, 2005
		Cow, Sheep, Goat	Lopez-Calleja *et al.*, 2005
		Pork	Rodriguez *et al.*, 2005
6	D-loop	Pig	Takeda *et al.*, 1995; Montiel-Sosa *et al.*, 2000
		Wild animals	Prakash *et al.*, 2000
		Many species	Bellis *et al.*, 2003
		Dog, Cat	Halverson and Basten, 2005
		Wild animals	Malisa *et al.*, 2006
7	$tRNA^{Glu}$, cyt-b gene	Many species	Wolf *et al.*, 1999
8	Cyt oxidase I gene	Cattle	Tartaglia *et al.*, 1998
9	$tRNA^{Lys}$-ATPase 8	Ruminants	Kingombe *et al.*, 2001; Feligini *et al.*, 2007

3 Materials & Methods

Animal species identification has been an area of concern in the milieu of social, religious, economic, forensic and health reasons. There have been many techniques developed for this purpose, but each technique has its own advantage and disadvantages. However, the PCR-based molecular techniques are being considered best amongst all others. The present investigation was undertaken with the aim to develop PCR based molecular techniques particularly species-specific PCR assays and multiplex PCR for the identification of animal species.

3.1 Species analyzed

In the present study, meat as well as blood samples from cattle, buffalo, camel, horse, sheep, goat, pig, dog, tiger, leopard, barking deer or muntjak, sika deer, goral, sambar, elephant and human (Table 3.1) were collected for standardization of DNA based molecular techniques for species identification. In addition, samples from avian species viz., black kite, rose-ringed parakeet, chicken (White Leghorn, Columbian, Aseel, Kadaknath, Australorp, Rhode Island Red, White Cornish, New Hampshire and cross-bred), duck (Vigova Super and Khaki Campbell), Turkey, Guinea fowl (Pearl and Lavender) and Japanese quail. Also, rabbit (*Oryctolagus cuniculus*) and five species of cold water fishes (*Tor putitora, Schizothorax richardsonii, Raiamas bola, Hypophthalmichthys molitrix* and *Garra gotyla gotyla*) were also collected for species differentiation (Table 3.1).

Table 3.1: Details of sample collection from various locations

Species	Species (Latin) name	Samples collected	Place of collection
1. Cattle	*Bos indicus* and *Bos taurus*	52	Instructional dairy farm, Veterinary clinics and Post-mortem hall, Pantnagar; Veterinary hospitals, Karnataka
2. Buffalo	*Bubalus bubalis*	46	Kiccha/Bareilly market, Instructional dairy farm, Pantnagar. Veterinary hospitals, Karnataka
3. Sheep	*Ovis aries*	37	Kiccha and Bareilly market, experimental animals, Pantnagar
4. Goat	*Capra hircus*	42	Kiccha and Bareilly market, Experimental animals, C.V.A.Sc, Pantnagar
5. Pig	*Sus scrofa domesticus*	34	Rudrapur and Pantnagar local markets
6. Camel	*Camelus dromedarus*	1	Veterinary college, Anand, Gujarat
7. Horse	*Equus caballus*	15	Army stud farm, Haldwani
8. Dog	*Canis familiaris*	40	Clinics- Veterinary College Pantnagar and Bangalore
9. Rabbit	*Oryctolagus cuniculus*	10	Experimental animal house, C.V.A.Sc, Pantnagar
10. Chicken	*Gallus gallus*	58	Local market, Pantnagar Instructional poultry farm, Pantnagar
11. Duck	*Anas platyrhynchos*	10	College of Fisheries, Pantnagar
12. Turkey	*Meleagris gallopavo*	4	Instructional poultry farm, Pantnagar
13. Guinea	*Numida meleagris*	30	Instructional poultry farm, Pantnagar
14. Japanese quail	*Coturnix japonica*	10	C.A.R.I, Izatnagar, Bareilly
15. Tiger	*Panthera tigris*	2	Pandit G.B. Pant High Altitude Zoo, Nainital
16. Leopard	*Panthera pardus*	3	
17. Barking deer	*Muntiacus muntjak*	3	
18. Sika deer	*Cervus nippon*	3	
19. Goral	*Naemorhedus. goral*	2	
20. Kite	*Milvus migrans*	2	
21. Parakeet	*Psittacula krameri*	1	
22. Sambar	*Cervus unicolor*	1	Veterinary clinics, Pantnagar
23. Elephant	*Elephas maximus*	8	Corbett Tiger reserve, Ramnagar
24. Human	*Homo sapiens*	1	Volunteer
25. Fish	*Tor putitora, Schizothorax richardsonii, Raiamas bola, Hypophthalmichthys molitrix* and *Garra gotyla gotyla*	10	NRC on Cold Water Fisheries, Bhimtal

3.2 Collection and transportation of samples

Approximately, 50-500 g tissue samples from these animal species were collected from the local markets, post mortem halls and veterinary clinics (Table 3.1). In cases where collection of tissue samples was not feasible; the blood, milk or semen samples were collected. Animals that have been prohibited for slaughter and protected under law (wildlife) approximately 2-10 ml of venous blood was collected from jugular/coccygeal and in case of birds wing vein into a sterile polypropylene centrifuge tube containing EDTA (0.2 ml, 0.5 M) as anticoagulant and mixed well (heparin was avoided since it is a known PCR inhibitor).

The samples were transported to the laboratory under chilled conditions (~4°C) and were then stored in either refrigerator (at 4°C) or in deep freezer (-20°C) until processing.

3.3 Chemicals/reagents/plastic wares

All the chemicals and reagents used in the present study were of molecular biology grade procured either from M/s Promega, Novagen, Fermentas, Life-sciences, Imperial life sciences, Bangalore Genei or Merck (Bioron, Bioserve). However, the plastic wares were mostly obtained from M/s Axygen and Tarsons.

3.4 Scientific equipment

Electronic balance (Sartorius), digital pH meter (Merck), variable volume (adjustable) micropipettes (Nichipet, Tripette and Eppendorf), refrigerated centrifuge (Mikro-2000R, Hettich and Haereus), UV/VIS spectrophotometer (Lambda 35, Perkin Elmer), submarine gel electrophoresis unit (Thermo), gel documentation system (Alpha Innotech), thermal cycler (GeneAmp PCR system 9700, Applied Biosystems), water bath (GFL, Merck, Germany), dry bath (Bangalore Genei), deep freezer -20°C (BlueStar), refrigerator (LG, Godrej), homogenizer (Polytron), Micro-oven (LG), etc were the routine laboratory equipment used for the study.

3.5 Isolation of DNA

3.5.1 Isolation of DNA from tissue/ meat samples

DNA extraction from tissue samples was undertaken using Wizard® Genome DNA purification kit (Promega, Madison, USA) following the manufacturer's instructions supplied along with the kit. Briefly, 20-100 mg of tissue was cut into very small pieces using a pair of scissors and placed in a 1.5 ml micro centrifuge tube. A master mix (275 µl) consisting of 200 µl of nuclei lysis solution, 50 µl of 0.5 M EDTA (pH 8), 20 µl of proteinase K (20 mg/ml) and 5 µl of RNase A solution (4 mg/ml) was added to each tube. The tubes were then incubated for 16-18 hrs at 55°C. After incubation, 250 µl of Wizard® SV lysis buffer was added to the tubes and then vertexed. The contents were loaded onto Wizard® SV mini column assembly and centrifuged @ 10,000 rpm for 3 minutes. The mini columns were then washed with 650 µl of Wizard® SV wash solution 4-6 times, dried by centrifugation for 2 min and then transferred to fresh 1.5 ml tubes. A volume of 250 µl of nuclease free water (NFW) was added and incubated for 2 min at room temperature and centrifuged for 2 minutes at the same speed for 1 min. An additional 250 µl of NFW was added to the tubes and incubated for 2 min. after incubation, the tubes were centrifuged for 2 min and the pooled elutes were collected and stored at -20°C for further use.

Alternatively, Phenol: Chloroform method (Sambrook and Russel, 2001) was used for the extraction of DNA from the tissue samples with modifications. The tissue samples (0.2-1 g) were cut into very small (<1 mm) pieces or pulverized in liquid nitrogen and 10 volumes (w/v) of DNA lysis buffer (pH 8) containing Ribonuclease-A @ 100 µg/ml (20 µg/ml) was added and incubated at 37°C for 1 h. Then, proteinase-K solution (20 mg/ml) was added @ 200 µg/ml and again incubated at 50°C for not less than 3 h or overnight. During incubation, regular swirling of the tubes was undertaken gently from time to time. Equal vol. of Tris-saturated phenol (equilibrated with 0.1 M Tris-Cl, pH 8.0) was added and the contents of the

tubes were subjected to mixing end to end for 10 min. and the contents were then centrifuged at 6,500 RPM for 15 min. The upper aqueous phase was collected into a fresh tube and equal volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture was added and centrifuged. The upper layer was collected and again equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and centrifuged. Finally, the upper aqueous phase was collected in a fresh tube and equal volume of chloroform was added and then centrifuged. The upper phase was again collected in to a fresh tube containing 0.2 volumes of 10 M ammonium acetate and 2 volumes of absolute ethanol and was mixed well for precipitation of DNA. The mixture containing visible DNA threads were centrifuged at (10,000 RPM for 10 min). The DNA pellet was washed twice with 70% alcohol by centrifugation (10,000 RPM for 5 min each), dried over a dry bath at 60°C and then dissolved in 1X TE (Tris-EDTA) buffer (50- 100 µl) or nuclease free water either. These DNA samples were used for PCR or stored at -20°C until further use.

3.5.2 Thermal processing of the tissue samples

The raw meat samples were subjected to thermal processing at different temperatures for a fixed time so as to assess detection ability of the diagnostic tool (PCR). For this purpose, fresh meat samples (or frozen or samples thawed to room temperature) were cooked in boiling water bath/ dry bath at 60°C, 80°C, and 100°C for 30 minutes. To simulate the steam cooking under pressure, autoclaving was done at 121°C for 30 minutes. In addition, micro oven cooking was also performed and duration of cooking varied upon the size of the meat cuts to ensure proper cooking. From these samples, DNA was extracted as per methods described in Section 3.5.1

3.5.3 Preparation of meat mixtures

Initially, a base adulteration mixture (BAM) was prepared which consisted of tissue (meat) of other species in equal proportion (20 mg each) except the one which is to be

analysed (Table 3.2). Only 6 major species i.e., cattle (Ca), buffalo (B), sheep (S), goat (G), pig (P) and chicken (Ch) were considered for this purpose. Base adulteration mixture was separately mixed with varying percentage of species specific meat tissue (to be analysed) ranging from 0.1% to 10% (Table 3.3). A total of 100 mg meat mixture from each sample was then subjected to DNA isolation using Wizard kit as described in Section 3.5.1.

Table 3.2: Preparation of base adulteration meat mixture (in mg)

Meat	Ca-less BAM	B-less BAM	S-less BAM	G-less BAM	P-less BAM	Ch-less BAM
Cattle		20	20	20	20	20
Buffalo	20		20	20	20	20
Sheep	20	20		20	20	20
Goat	20	20	20		20	20
Pig	20	20	20	20		20
Chicken	20	20	20	20	20	
Total	100	100	100	100	100	100

Table 3.3: Preparation of adulteration meat mixtures (in mg)

Adulteration level	Cattle (Ca)		Buffalo (B)		Sheep (S)		Goat (G)		Pig (P)		Chicken (Ch)	
	Ca-less BAM	Ca	B-less BAM	B	S-less BAM	S	G-less BAM	Goat	P-less BAM	P	Ch-less BAM	Ch
0.1%	99.9	0.1	99.9	0.1	99.9	0.1	99.9	0.1	99.9	0.1	99.9	0.1
0.5%	99.5	0.5	99.5	0.5	99.5	0.5	99.5	0.5	99.5	0.5	99.5	0.5
1%	99.0	1.0	99.0	1.0	99.0	1.0	99.0	1.0	99.0	1.0	99.0	1.0
5%	95.0	5.0	95.0	5.0	95.0	5.0	95.0	5.0	95.0	5.0	95.0	5.0
10%	90.0	10.0	90.0	10.0	90.0	10.0	90.0	10.0	90.0	10.0	90.0	10.0

3.5.4 Isolation of DNA from meat products

Different meat products were prepared/ procured from local market/veterinary college/research institutes and DNA was isolated as described in the section 3.5.1. The meat products such as buffalo meat kabab(2), mutton curry (2), chevon curry (2), pork sausage (2), chicken patties (2), samosa (2), nuggets (2) and loaves (2) were also subjected to DNA isolation using Wizard genome DNA purification kit.

3.5.5 Isolation of DNA from the blood samples

The DNA from blood samples was isolated by using either Fermentas purification kit or by phenol: chloroform method (Sambrook and Russel, 2001) with slight modifications. Fresh blood (20 ml) samples were collected in 3.5 ml ACD or EDTA and centrifuged (2,500 rpm for 15 min). The supernatant was discarded and buffy coat was collected. To this about 15 ml lysis buffer was added and the tubes were incubated at 37°C for 1 hr. DNA isolation was continued as described in Section 3.5.1.

The frozen blood samples were thawed to room temperature in water bath and equal volume of phosphate buffered saline (PBS) was added. The tubes were properly swirled and centrifuged (5,400 rpm for 15 min, 25°C). The supernatant was discarded and the pellet was collected. To this, 15 ml lysis buffer was added and incubated at 37°C for 1 hr. Thereafter, the steps mentioned in section 3.5.1 were followed for the extraction of DNA.

3.5.6 Isolation of DNA from semen samples

Diluted/extended semen samples (0.2 ml, French mini Artificial Insemination straws) were procured from cattle (12) and buffalo (4) and transferred to 1.5 ml micro centrifuge tube and DNA was extracted using Wizard® SV Genomic DNA Purification System as described in **Section 3.5.1**

3.6 Checking the quality, purity and concentration of DNA

The quality of isolated DNA was checked by agarose gel (0.6-0.8%, w/v) electrophoresis. About 5 µl of dissolved DNA (in TE buffer or NFW) was mixed with 1 µl of 6X gel loading dye (Xylene Cyanol and Bromophenol Blue) and loaded on to a well along with a 1 kb molecular weight marker. Electrophoresis was undertaken @ 5-8 V/cm and ethidium bromide (added @ 0.5 µg/ml in the agarose) stained DNA bands were visualized under UV trans-illuminator and documented over a gel documentation system to assess the quality of isolated DNA.

The purity of the isolated DNA was checked by using UV/ VIS spectrophotometer. The DNA samples with OD_{260}:OD_{280} ratios between 1.7 and 1.9 were used for PCR amplification.

The concentration of the DNA was estimated by using following formula-

$$\text{DNA concentration (µg/ml)} = \frac{OD_{260} \text{ x Dilution Factor x } 50}{1000}$$

(1 OD value at 260 nm is equivalent to ~50 µg of dsDNA / ml)

3.7 Species-specific PCR assays

Species-specific PCR assays were standardized for 6 meat species i.e., cattle, buffalo, sheep, goat, pig and chicken.

3.7.1 Design of species-specific primers

The mitochondrial (mt D-loop) and nuclear (5-aminolevulinate synthase gene, a repetitive SINE) DNA sequences were downloaded from the GenBank database and aligned using "*Megalign*" software (DNAStar, Inc., 1996). The primer sites with nucleotide sequence variation between the species and homology within a species (free from SNPs) were located. The oligonucleotide primers were designed against such unique species-specific sites so as to yield appropriate sized PCR products; and the primers were designed using "*Primer-Select*" software (DNAStar Inc., 1996). Further, the selected primers were screened for primer specificity (species-specificity) and cross reactivity using local alignment tool "*BLAST*" (http://www.ncbi.nlm.nih.gov/blast). Finally, selected primers were custom synthesized from IDT, Germany and Bioserve, Hyderabad, and they were considered for PCR amplification in a given species (Table 3.4, Fig 3.1-3.6).

Table 3.4: Species-specific oligonucleotide primers

Species	Target	Primer	Direction	Sequence	Primer size	Tm ^{o}C	Spp. Specific amplicon
Cattle	mt D-loop	VPH-CF	Forward	5'-TAT CAA AAA TCC CAA TAA CTC AAC ACA-3'	27 bp	52.0	384 bp
		VPH-CR	Reverse	5'-GGG CCC GGA GCG AGA AG-3'	17 bp	61.6	
Buffalo	mt D-loop	VPH-BF	Forward	5'-TAG AAA TAA CTG CAA CCA TCA ACA C-3'	25 bp	53.0	534 bp
		VPH-BR	Reverse	5'-GTC CAA GCA TCC CCC AAA AT-3'	20 bp	52.0	
Sheep	mt D-loop	VPH-SF	Forward	5'-CCA CCC ACG GAC ACG AG-3'	17 bp	58.5	329 bp &
		VPH-SR	Reverse	5'-AGT TCA ATG CCC TAT ATG CTT CAG-3'	24 bp	55.0	404 bp
Goat	mt D-loop	VPH-GF	Forward	5'-TCC CAC TCC ACA AGC TTA CAG ACA-3'	24 bp	59.9	436 bp
		VPH-GR	Reverse	5'-GCT TAT ATG CAT GGG GGA GAT-3'	21 bp	54.5	
Pig	mt D-loop	VPH-PF	Forward	5'-AAT TTT TGG GGA TGC TTA GAC T-3'	22 bp	52.5	712 bp
		VPH-PR	Reverse	5'-TAT TTT GGG AGG TTA TTG TGT TGT A-3'	25 bp	52.6	
Chicken	5-amino	VPH-ChF	Forward	5'-CCG GCA GCA GAG CAG ACT AAC AAC-3'	24 bp	62.0	288 bp
	levulinate	VPH-ChR	Reverse	5'-AGG GGA TAC GCC GAC TGC TGA G-3'	22 bp	63.1	
	sythase gene						

3.7.2 Optimization of the species-specific PCR

The PCR conditions i.e., primer concentration, dNTPs, $MgCl_2$, and the cycling programme especially the annealing temperature were standardized for each species.

The optimized PCR was set up in a 25 µl volume reaction mixture consisting of 2.5 µl of 10X assay buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM $MgCl_2$, Bioron, GmbH], 0.5 µl (200 µM each) of dNTP mix [Sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total pH 7.5, Promega, USA], 0.5 µl (20 Pico moles) each of forward and reverse primers (Integrated DNA Technologies-IDT, Coralville, IA, USA/ Bioserve, Hyderabad), 1U Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron, GmbH, Germany), 50 ng of purified DNA and NFW (Merck, Germany) to make the volume. The tubes were flash spun and the PCR was performed in a Thermal cycler (Gene AMP® PCR System 9700, Applied Biosystems).

The cycling conditions consisted of an initial denaturation (95°C for 5 min) followed by 30 cycles of denaturation (95°C, 30s), primer annealing (55°C, 30s) and extension (72°C, 30s). After the final extension (95°C, 5 min), the PCR products were held at 4°C until electrophoresis.

Agarose gel (2%) was prepared in 1X TBE buffer and the PCR products (5 µl) stained with 6X gel loading dye (1 µl) were electrophoresed at 40-60 V for 1-3 hrs. The amplified products were visualized and confirmed over a gel documentation system (AlphaImager^R HP. Alpha Innotech corp.). The relative molecular weight of the amplicons was calculated against a 100 bp ladder (Fermentas).

3.7.3 Sensitivity of the species-specific PCR

The sensitivity of the species-specific PCR was tested for individual species. A 10-fold serial dilution of the template DNA starting from 10 ng downwards (10 ng, 1 ng, $1x10^{-1}$,

$1x10^{-2}$, $1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$, $1x10^{-6}$, $1x10^{-8}$, $1x10^{-9}$ and $1x10^{-10}$ ng/reaction) were prepared and the PCR was performed as described in the above sections.

3.7.4 Specificity/selectivity of the species-specific PCR

The specificity or selectivity of the species-specific PCR was evaluated using non-target species DNA. Cross-amplifications in non-target species was ruled out for each species studied using the standardized PCR. For excluding cross amplification and for the purpose of statistical analysis several breeds of animal were studies such as- cattle (Sahiwal, Holstein Freisian, Jersey, Deoni, Khillari and cross-bred), buffalo (Murrah), sheep (Muzaffarnagari), goat 1 (Black Bengal), pig (Large White Yorkshire), camel (Jaiselmeri), horse (Through bred), dog (12 breeds), rabbit (Newzealand white), chicken (White Leghorn, Columbian, Aseel, Kadaknath, Australorp, Rhode Island Red, White Cornish, New Hampshire and a cross-bred i.e., synthetic broiler), duck (Vigova Super and Khaki Campbell), turkey (2 breeds), guinea fowl (pearl and lavender), Japanese quail, tiger (Royal Bengal and Siberian), leopard, barking deer, sika deer, sambar, goral, kite, parakeet, elephant, human and 5 species of fish.

3.7.5 Statistical analysis in species-specific PCR

Two-row by Two-column (2 x 2) Contingency Table was used to compare the species-specific PCR vs. the phenotype of the animal species (**Table 3.5**).

Table 3.5: Application of 2x2contingency table in the animal species identification

Phenotype	**PCR**		
	Positive	**Negative**	∑
Positive	**a (True positive)**	**b (False Negative)**	**a+b**
Negative	**c (False Positive)**	**d (True Negative)**	**c+d**
∑	**a+c**	**b+d**	**a+b+c+d**

Various derived formulae used for the analysis in the intra-laboratory validation study are shown below (these could also be used for the inter-laboratory validation studies).

Where,

a- Number of samples (meat) conforming to an animal species of animals based on phenotypic appearance and also were positive by PCR i.e., True Positive.

b- Number of samples (meat) that not-conforming to an animal species based on phenotypic appearance but were positive by PCR i.e., False Positive.

c- Number of samples (meat) that conforming to an animal species based on phenotypic appearance but were negative by PCR i.e., False Negative.

d- Number of samples (meat) not-conforming to a specific animal species based on phenotypic appearance and were also negative by PCR i.e., True Negative.

- **Inclusivity (%) =** $\frac{a}{(a+b)} \cdot 100$

Inclusivity is the ability of an alternative method (PCR) to detect the target species from a wide range of breeds or strains.

- **Exclusivity (%) =** $\frac{d}{(c+d)} \cdot 100$

Exclusivity is the lack of response from a relevant range of closely related but non-target species.

Selectivity is the measure of the degree of response from target and non-target species and it comprises of **inclusivity** and **exclusivity**.

- **Positive predictivity (%) =** $\frac{a}{(a+c)} \cdot 100$

- **Negative predictivity (%) =** $\frac{d}{(b+d)} \cdot 100$

- **Analytical accuracy (%) =** $\frac{a+d}{(a+b+c+d)} \cdot 100$

Analytical Accuracy includes primary validation on the known pure breeds for the establishment of selectivity and detection probability.

- **Kappa index =** $\dfrac{2(ad-bc)}{(a+c)\,.\,(c+d)+(a+b)\,.\,(b+d)}$

The **Kappa index** indicates the strength of relationship between the rows and columns variables of a cross tabulation (Sachs 1984, Malorny *et al.,* 2003). Kappa values were read as follows,

- Ø <0.01 indicates no concordance.
- Ø 0.10-0.40 indicates weak concordance.
- Ø 0.41-0.60 indicates clear concordance.
- Ø 0.61-0.80 indicates strong concordance.
- Ø 0.81-1.00 indicates nearly complete concordance.

3.8 Multiplex PCR

A multiplex PCR was standardized for the simultaneous detection of cattle and buffalo meat. For this purpose, species-specific forward i.e., cattle (5'-TAT CAA AAA TCC CAA TAA CTC AAC ACA-3') and buffalo (5'-TAG AAA TAA CTG CAA CCA TCA ACA C-3') specific primers and a common reverse (5'-GGG CCC GGA GCG AGA AG-3') primer were used. The PCR was performed using the same conditions as described in Section 3.7.2

3.9 Identification of animal species using universal primers

Six species i.e, of sika deer, leopard, muntjak, parakeet, black kite and camel were identified by the sequence analysis of mt 12 S rRNA gene.

3.9.1 PCR amplification of mt 12S rRNA gene

For the identification and confirmation of the unknown species such as wild animals, the DNA was isolated from the blood samples as described previously (Section 3.5) and PCR

was performed using universal primers (Kocher *et al*., 1989) against the flanking sequence of mt 12S rRNA gene of sika deer (*Cervus nippon*), leopard (*Panthera pardus*), muntjak (*Muntiacus muntjak*), parakeet (*Psittacula krameri*), black kite (*Milvus migrans*), and camel (*Camelus dromedarius*)

PCR was performed in a 50 µl reaction volume; consisting of 5 µl of 10X Assay buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM $MgCl_2$ (Bioron, GmbH)]; 1 µl (200µM each) of dNTP mix [sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total pH 7.5 (Promega, Madison, WI USA)], 1µl or 20 Pico moles each of forward (5'-CAA ACT GGG ATT AGA TAC CCC ACT AT-3' 26 mer) and reverse (5'- GAG GGT GAC GGG CGG TGT GT-3' 20 mer) primers (Bangalore Genei); 1.66 U Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron GmbH, Germany), 50 ng of purified DNA and autoclaved NFW to make the final volume. The tubes flash spun and the PCR was performed using a Thermal cycler (Gene AMP® PCR System 9700, Applied Biosystems).

The cycling conditions involved an initial denaturation (94°C for 5 min) followed by 30 cycles of denaturation (94°C, 45s), primer annealing (60°C, 45s,) and extension (72°C, 1 min); after the final extension (72°C, 5 min), the PCR products were held at 4°C. Agarose gel (2% in 1x TBE and 0.5 µg/ml ethidium bromide) electrophoresis (50 V, 2 hrs) was performed and the amplified products were visualized under UV illumination and the gel was documented.

3.9.2 Sequencing of PCR products from mt 12S rRNA gene

The PCR products were sequenced using ABI automatic DNA sequencer (3100/ABI3100-1699-013) at a DNA sequencing facility (Bangalore Genei, Bangalore). The sequences obtained were aligned using '*Megalign*" and compared by '*Clustal*' method

(DNAstar, Inc. 1996). The nucleotide sequences were then submitted to the GenBank/ EMBL nucleotide sequence database.

3.9.3 BLAST analysis of mt 12S rRNA gene sequence

1. Logged on to the NCBI site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).
2. Nucleotide blast (*BlastN*) was selected.
3. The unknown sequence (Accession number, gi, or FASTA) was entered.
4. Nucleotide collection and highly similar sequences (*Megablast*) option were selected.
5. The sequence was subjected to Blast.
6. The data retrieval was in two modes, i.e., distance tree and alignments.

(a) Distance tree of results: Searched by sequence ID, taxonomic name, sequence title or the Blast name. Further, a focused sub-tree as well as the alignment report of the desired species (or accessions) was browsed.

(b) In '*Alignments'* option, the sequence show significant alignments along with their accession numbers, title, score, E (bits) value, followed by the detailed information about the length of sequence, percent identity, gaps, etc.

Based on the distance tree and alignments with the query sequence, the nearest species with the maximum score was found and the animal species was identified.

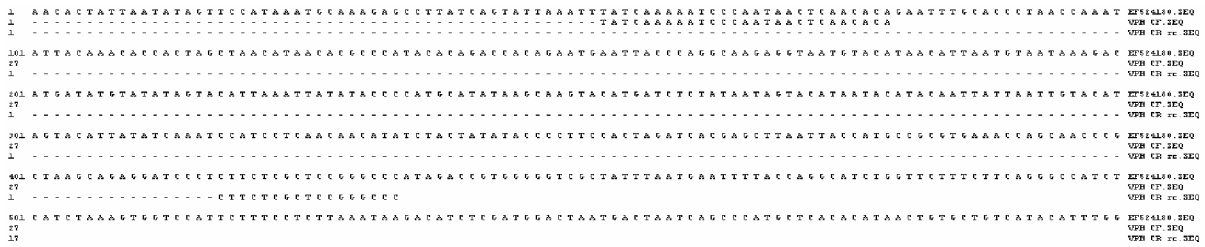


Fig 3.1 Alignment of cattle specific primers (VPH CF & CR) to mt D loop region

Fig 3.2 Alignment of buffalo specific primers (VPH BF & BR) to mt D loop region

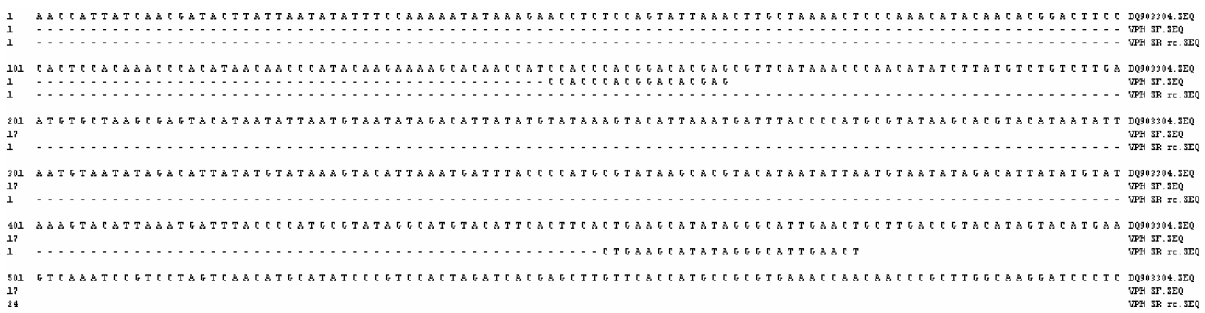


Fig 3.3a Alignment of sheep specific primers (VPH SF & SR) to mt D loop region

Fig 3.3b Alignment of sheep specific primers (VPH SF & SR) to mt D loop region

Fig 3.4 Alignment of goat specific primers (VPH GF & GR) to mt D loop region

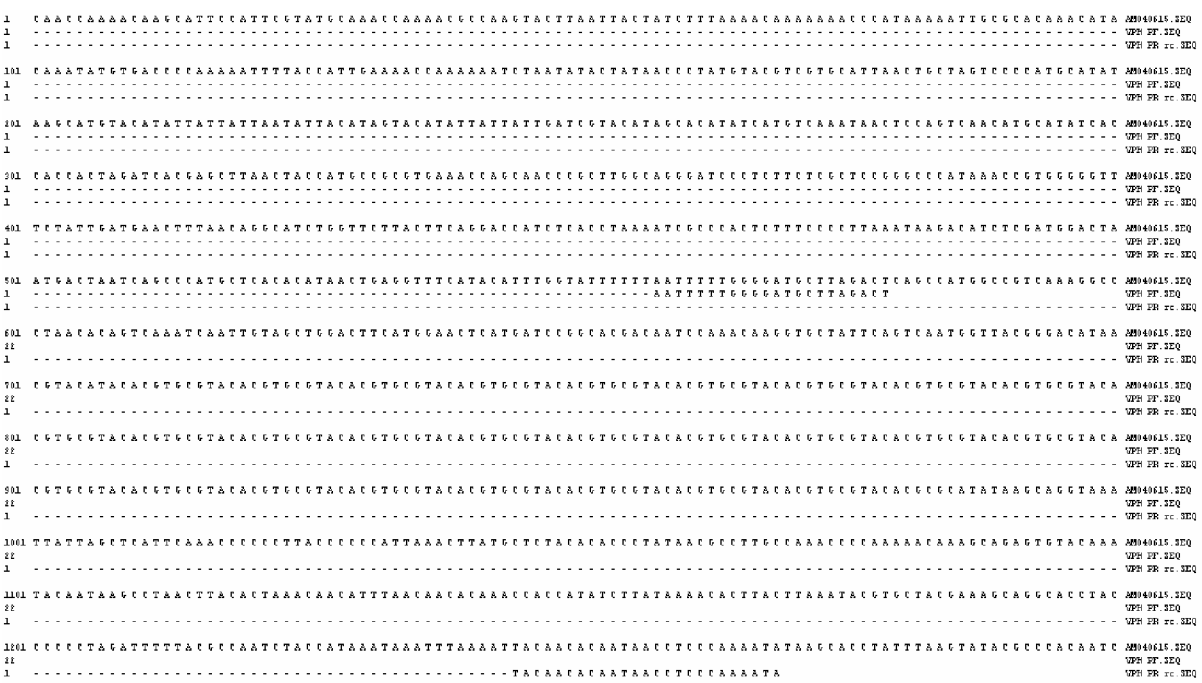


Fig 3.5 Alignment of pig specific primers (VPH PF & PR) to mt D loop region

Fig3.6 Alignment of chicken specific primers (VPH ChF & ChR) to chicken 5-amino levulilinate synthase gene

4 Results & Discussion

The present investigation was undertaken with the objective of developing simple and specific PCR based molecular diagnostic techniques for the identification of major meat animal species. The technique involved development of species-specific PCR assays for the precise identification of cattle, buffalo, sheep, goat, pig and chicken species and to use these assays for the detection of particular species in a meat mixture at different levels of adulteration. This task was accomplished by PCR amplification of mitochondrial (mt D loop) and nuclear (5-amino levulinate synthase gene) DNA. Further, for the identification of the species other than above mentioned six food animals, an alternate technique was employed, which involved sequence analysis of mitochondrial 12S rRNA gene. A total of 25 animal species (consisting of different breeds) were considered for this purpose (Table 3.1).

4.1 DNA extraction

In the present study, two approaches of DNA isolation i.e., a spin column based technique using Wizard DNA purification kit and a classical phenol-chloroform technique (Sambrook and Russel, 2001) were used. The Wizard^{TM} DNA purification kit involved use of spin columns, which reduced the losses in the DNA yield that occurred during the separation of aqueous and organic phases of conventional phenol-chloroform method. Also, avoidance of DNA handling eliminated the chances of shearing of intact DNA. Further, use of mini-columns helped in the preparation of adulteration mixtures using a fixed amount of sample. Both the methods yielded comparable total DNA in raw as well as thermally processed tissue samples (Table 4.1).

The samples with OD_{260}/OD_{280} ratio between 1.7 and 1.9 were considered for the PCR amplification. Although DNA gets degraded into small fragments at high temperatures,

but still amplification of target region was found satisfactory. The PCR amplification was thus possible even in the samples exposed to autoclaving temperatures of 121°C. Further, it was noticed that upon thermal processing of the samples, there was a rise in the quantity of DNA (yield). This rise might be probably due to the dehydration process that would have increased the number of cells per unit weight of the tissue. Similar finding were also observed by other investigators (Fairbrother *et al.,* 1998 and Girish, 2003).

4.2 Development of species specific PCR assays

4.2.1 Species-specific PCR for the specific identification of animal species

4.2.1.1 Species-specific PCR for cattle

Species-specific primers for cattle mitochondrial D loop (size 911 bp) were designed that flanked sequence (Accession no. EF524180) between base positions 53 to 434 yielding a specific product of 381 bp (Accession no. FM179472). This sequence was found common for both *Bos indicus* and *Bos taurus* enabling the identification of all the indigenous, exotic as well as cross-bred cattle (Fig 4.1, Table 4.2 & 4.3). Further, PCR amplification was confirmed in the six breeds of cattle i.e., Sahiwal, Holstein Frisian, Jersey, Deoni, Khilari and cross-bred.

In this study, 0.1 pg of template DNA was detected which indicated a very high sensitivity of the technique compared to that of conventional PCR methods for the detection of cattle meat. Although PCR offers remarkable sensitivity, some authors consider it undesirable to have detection limit below 0.1% (Meyer *et al.*, 1995).

Many investigators have identified cattle meat using PCR with a limit of detection (LOD) ranging from 2.5 to 250 pg (Tartaglia *et al.,* 1998; Matsunaga *et al.,* 1999a & b; Bellagamba *et al.,* 2001; Kremar and Rencova, 2001 and Brodmann and Moor, 2003). It is worth to mention that these authors used different targets and primer sequences. In view of

the limit of detection obtained in the present study, it appears that the PCR assay was more sensitive than those reported earlier. However, Walker *et al.* (2003) and Lopez-Andreo *et al.* (2005), detected 0.1 pg and 0.06 pg bovine DNA respectively, using real time PCR.

4.2.1.2 Species-specific PCR for buffalo

In buffalo, a pair of species-specific primers for the mitochondrial D loop (size 1,015 bp) were designed which amplified a discrete product of 534 bp (Accession no. FM179473) flanking between positions 168 and 701 (Fig 4.2). Testing the primers through BLAST analysis of NCBI revealed that the common primer sequence could be used for all the water buffalo breeds.

Malisa *et al* (2006) also used PCR amplification of mt D-loop for identification of buffalo meat species but with different primer sequences. Apart from PCR amplification of mt D loop for buffalo meat species identification, Rajapaksha *et al* (2003) and Girish *et al* (2005) have exploited 12S rRNA and mt cyt b gene, respectively for their specific amplification. The technique could differentiate buffalo from other related animals based on the molecular weight of the PCR product. Interestingly, the buffalo specific PCR was found highly specific to buffalo in the present study. It showed fairly good sensitivity (LOD 10 pg) even in the cooked and adulterated meats.

4.2.1.3 Species-specific PCR for sheep

Like other species of food animals, single pair of sheep specific primers was used in the present study. Four PCR products were obtained from mt D loop (size 1,260 bp) in raw meat. However, only two amplicons with the size of 329 bp (position 148-476 bp of DQ903304) and 404 bp (148-551 bp of DQ903233) were found in cooked as well as adulterated meat samples. This phenomenon could be due to ‘heteroplasmy’ meaning occurrence of ≥2 types (copy number) of mitochondria. Malisa *et al.* (2006) also reported similar phenomenon appearing in many animal species including sheep. It was observed that

as the template concentration (target copies) went on reducing, the least common copies got exhausted first leaving behind the more abundant copies. Out of four only two PCR amplicons (from the abundant copies) of molecular weight 329 bp and 404 bp were considered diagnostic as these amplicons were seen even in the autoclaved and adulterated meat samples (Fig 4.3).

4.2.1.4 Species-specific PCR for goat

Of the 1,212 bp caprine mt D loop (Accession no. DQ121616) sequence, a fragment of 436 bp (position 98-533) was amplified using caprine specific primers (Accession no. FM179474). The upper primer annealed between positions 98-121 and the lower one at 513-533 bp (Fig 4.4).

Previously, PCR-RFLP has been used for the identification of goat using different restriction enzymes after the PCR amplification of specific targets such as cyt b gene (Partis *et al.,* 2000), mt 12S rRNA gene (Girish *et al.,* 2005) and mt D loop (Malisa *et al.,* 2006). Based on the specific amplification of mt 12S rRNA gene, Lopez-Calleja *et al.* (2005) identified goat and clearly differentiated it from the sheep. Similarly, Chikuni *et al.* (1994a) identified sheep and goat and later differentiated them. The goat specific assay developed in the present study offered several advantages over the previously reported techniques as there was no need to apply RFLP for goat meat identification. The goat specific PCR product was observed only in goat sample. The newly developed assay was found highly sensitive (LOD 0.1 pg) and was capable of detecting even the cooked and adulterated meat samples.

4.2.1.5 Species-specific PCR for pig

Detection of pork by PCR has been reported previously by targeting nuclear (Meyer *et al.,* 1994; 1995; Meer and Eddinger, 1996) as well as mitochondrial DNA sequences (Montiel-Sosa *et al.,* 2000). The sequence of pig mt DNA has also been reported (Ghivizzani *et al.,* 1993 and Urshing and Arnason, 1998). Highly species-specific primers have been

designed for the detection of pork and fat targeting pig D loop mt DNA (Montiel-Sosa *et al.,* 2000), where a pork specific PCR product of 531 bp was obtained in pork as well as wild boar. During the course of present study, the newly designed pig specific primers amplified a portion of mt D loop (1,105 bp) from base positions 448 to 1269 bp (Accession no. AM040615) which gave a single pig specific amplicon of 712 bp in length (Fig 4.5).

The pig-specific PCR developed in this study offered certain advantages over the previously reported methods- (i) developed pig-specific PCR assay did not require any further RFLP or sequencing for the confirmation, as the specific product of 712 bp was evident only in pork and not in other species studied; (ii) sensitivity of the assay was 10 pg and detection of adulteration at the level of 0.1%. A pork-specific assay designed by Montiel-Sosa *et al.* (2000), could detect adulteration only up to the level of 5%. However, Walker *et al.* (2003) could record the effective minimum quantitation of 0.01 pg using real time PCR. Similarly, Calvo *et al*. (2001a) detected pork DNA up to the level of 1.25 pg in the meat products and the LOD in their assay was 10 pg. Even with real time PCR, Rodriguez *et al*. (2005) could record a sensitivity of 10 pg for the specific detection of pork, whereas Dooley *et al.* (2004) detected up to 0.5% of pork in the meat mixture.

Further, existence of sequence polymorphisms in D loop region could help in the discrimination of closely related species (Takeda *et al.,* 1995; Montiel-Sosa *et al.,* 2000). On the other hand, heterologous hybridization of pork mt DNA to cattle had been reported (Montiel Sosa *et al.,* 2000). To avoid such ambiguities a thorough screening of the primers and testing for the cross- amplification in the related species was undertaken in the present study; for which 25 species of animals were considered.

4.2.1.6 Species-specific PCR for chicken

For identification of chicken meat, species specific PCR was standardised in the present study. Nuclear 5-amonolevulinate (ALA) synthase structural gene (6.9 kb long

containing 10 exons) was targeted of which the exon 1-7 had a size of 5,282 bp (Accession no. X03517) with initial region between positions 6 and 293. After PCR amplification with these self designed species specific primers in chicken, a product of 288 bp (Table 4.2, Fig 4.6) was obtained.

The size of the PCR products were found similar in all the breed/strains of chicken tested, but certainly not in other related avian species such as turkey, duck, guinea fowl, quail, black kite and parakeet. The amplified products were sequenced and submitted to the EMBL database for Aseel (Accession no AM933753) and Kadaknath (Accession no AM933752) chicken breeds.

Further, chicken specific PCR assay could detect chicken DNA up to the level of 10 pg. However, using real time PCR and targeting the CRE-1 SINE of chicken; Walker *et al.,* (2003) detected 0.05% (5 pg) of chicken DNA in a mixture of other species. Similarly, 0.09 pg of chicken DNA was detected by Lopez-Andreo *et al.,* (2005). It was assumed that the level of detection of chicken DNA with species-specific primer used in this study would have increased many folds with the application of real time PCR.

Briefly, the species specific PCRs developed in the present study for all the species could exhibit optimum amplification of target DNA at a primer concentration of 20 pico moles and an annealing temperature of 55°C (Table 4.3).

Many mitochondrial sequences have been attempted as amplification targets such as cyt-b gene, cyt oxidase I gene, rRNA (12S, 16S, 18S, 28S) genes, D-loop, tRNAs ($tRNA^{Glu}$, $tRNA^{Lys}$-ATPases), ND5, etc by several investigators (Tartaglia *et al.,* 1998; Montiel-Sosa *et al.*, 2000; Dooley *et al.,* 2004; Girish *et al.*, 2004, 2005; Halverson and Basten, 2005; Lopez-Andreo *et al.*, 2005; Fumiere *et al.*, 2006; Feligini *et al.*, 2007 and Tanabe *et al.*, 2007 a & b) with variable results (Table 2.2). However, targeting of the mt D loop has been found promising as certain regions of mt D loop sequences are conserved in a species which help in

inter-specific and inter-generic comparison (Lockwood *et al.,* 1993). Further, the hypervariable region of mt D loop has been proved best for studies involving intra-species variation (Ferguson *et al.,* 1995; Hall and Nawrocki, 1995; Hansen and Loechcke, 1996) and thus help in designing primers against sequences that differ even within the species. Takeda *et al.* (1995) explored sequence polymorphism in the mt D loop region for the identification of different pig breeds. Similarly, wild felids were identified by amplifying mt D loop region using feline specific primers (Shankarnarayanan and Singh, 1998)

4.2.2 Effect of thermal processing of meat tissues on PCR amplification

4.2.2.1 Effect of processing on PCR amplification

The suitability of species-specific PCR assays was also evaluated in 20 raw tissue samples. These samples were subjected to microwave cooking as well as thermal exposure at 60°C, 80°C, 100°C and 121°C for 30 minutes. The diagnostic PCR signals were found in all the six species studied even up to the autoclaving temperature of 121^{o}C at 15 lbs pressure for 30 min (Table 4.4 and Fig 4.7-4.12).

In addition to the tissue samples, some meat products such as kabab, mutton curry, chevon curry, pork sausage, chicken patties, chicken samosa, chicken nuggets and chicken loaves were also analyzed using species-specific primers of particular species. It was interesting to note that the PCR amplification of these thermally processed meat products with respective species specific primers could detect the specific meat species. It was found that even the presence of oil and spices in the products or preparations did not affect the PCR amplification up to 121°C of processing temperatures. On the contrary, other investigators observed degradation of target DNA and loss of PCR signal in samples processed above 121°C (Ebbehoj and Thomsen, 1991a; Quinterio *et al.,* 1998; Buntjer *et al.,* 1999 and Brodmann and Moor, 2003).

Further, the wizard kit used in the present study for DNA isolation was found optimum in the thermally exposed (up to 121℃, 15 lbs pressure, 30 min) meat tissue as well as processed meat product samples. Since many copies of mt DNA D loop are available, even if some of the target genes get degraded at higher temperatures, still there would be few to yield the positive PCR signal (Girish, 2003) and thus mt D loop as a target qualifies for its suitability for PCR amplification.

4.4 Assessment of suitability of species-specific PCR in adulterated meat

The PCR amplification with species specific primers of all the six animal species was evaluated in the meat tissue adulterated with the closely related species. It was found that the species-specific PCRs developed in the present study were sensitive enough to identify meat adulteration up to the extent of 0.1% (Fig 4.13-4.18, Table 4.5)

Calvo *et al* (2002) targeted a repetitive element for the specific identification of beef in the meat products (sausages, canned food and hamburgers) with the detection of 1% beef adulteration in the cooked meat mixtures and 0.01% raw beef in pork. Similarly, other workers detected <1% beef in meat mixtures (Meyer *et al.,* 1995; Tartaglia *et al.,* 1998; Matsunaga *et al.,* 1999a & b; Calvo *et al.,* 2002b).

Species specific PCR assays developed in the present investigation could be used for the identification of cattle, buffalo, sheep, goat, pig and chicken even in the adulterated meat mixtures (up to 0.1%). Using these assay techniques only qualitative analysis of the meat mixture could be done .However for quantitative analysis, there is need to standardize and use of the real time PCR based assay.

4.2.3 Sensitivity of species-specific PCR

In the present study, the target DNA was subjected to ten fold serial dilution starting from 10 ng downwards and the PCR amplification was attempted. The last dilution giving the detectable amplification up on 30 PCR cycles (25 µl reaction volume) was considered as the

LOD. The sensitivity of the species-specific PCRs varied from 0.1 pg to 10 pg in different animal species i.e., 10 pg in buffalo, pig and chicken, 1 pg in sheep and 0.1 pg in cattle and goat (Table 4.3).

The sensitivity of PCR could be assessed in two ways either by serially diluting the target DNA whose concentration has been spectrophotometrically measured (Guoli *et al.* 1999, Tajima *et al.* 2002 and Mendoza-Romero *et al.* 2004) or by mixing the meat sample in different proportions into the non-target species (Tartaglia *et al.* 1998; Colgan *et al.* 2001; Krèmáø and Renèová 2001; Lahiff *et al.* 2001; Myers *et al.* 2001; Calvo *et al.* 2002; Lahiff *et al.* 2002; Kusama *et al.* 2004). In the present study, both the approaches were attempted; firstly, the meat sample was adulterated with meat mixture consisting of other species in equal proportions (Table 3.2 & 3.3) and secondly, the DNA concentration was measured spectrophotometrically and then diluted in different proportions in nuclease free water and tested by PCR for amplification for each species (Section 3.7.3). However, the first approach is most preferred since it simulates the practical/ field conditions.

It has been reported in the literature that the primers that amplify a short fragment of a target DNA give higher sensitivity (Guoli *et al.* 1999; Tajima *et al.* 2002; Walker *et al.* 2003 and Mendoza-Romero *et al.* 2004). Matsunaga *et al.* (1999a & b) amplified a 274 bp product of a mt DNA and recorded an LOD of 250 pg. Similarly, Frezza *et al.* (2003) amplified a 147 bp product with the sensitivity of 1 pg. However, in the present study, using species-specific primers although the product size was relatively large, the sensitivity was not all compromised (Table 4.3).

Further, the LOD of 0.1 pg for cattle and goat mt DNA, make these assays highly sensitive; although variable levels of LOD have been reported in the literature (Gouli *et al.*, 1999; Laube *et al.*, 2001; Tajima *et al.*, 2002; Walker *et al.* 2003; Mendoza-Romero *et al.*, 2004; Leipzig, 2005 and Tanabe *et al.*, 2007a & b). Brodmann and Moor (2003) opined that

the variation in the LOD from species to species could be due the variation in the copy number of the mitochondria between the species.

4.2.4 Specificity

The cross amplification from the closely related animal species was checked with all six species-specific PCR assays. For this purpose, the samples from 25 animal species consisting different breeds were subjected to PCR amplification to check the possibility of misidentification of the species (Fig 4.19-20).

When the targets were analyzed through the 'BlastN' of NCBI, all the primers showed their highest homology with the species for which they were designed. This indicated a very high level of primer specificity to the target sequence. Also, many closely related animal species were screened while designing these primers; hence the possibility of cross amplification was excluded both theoretically and practically. Although the mt D loop targets were specific to only the mammalian species studied, to rule out any nucleotide sequence homology and possible cross reaction contaminants were also tested by taking the DNA derived from plants and bacteria (*Salmonella* and *E. coli*).

4.3.5 Repeatability

The PCR amplification of target DNA with species specific primers was repeated 20 times where consistent results were recorded. It was interesting to observe that with constant PCR conditions and similar chemicals /reagents used in the PCR amplification, the constant and repeatable results were obtained. The repeatability was also assessed in the raw, cooked, as well as adulterated meat tissues along with some meat products similar findings.

4.3.6 Statistical analysis

The 2x2 contingency statistical analysis was performed by taking 20 samples from each species including the available breeds (Table 3.1). Only the unprocessed samples were considered for the intra-laboratory validation study (Table 4.6). However, no statistical

analysis of the meat samples indicating adulteration level ≤0.1% was performed. Ideally, the DNA should be extracted in 5 replicates from each adulterated meat sample and from each DNA sample, 5 PCR should be set up by taking template from every corner of the tube. It appears that such statistics has been applied for the first time in the animal species identification in the present study, where a phenotype *versus* PCR has been considered for testing the species-specific primers of the diagnostic PCR.

4.4 Multiplex PCR for simultaneous detection of cattle and buffalo

Differentiation of cattle and buffalo was undertaken by a multiplex PCR using species-specific forward and common reverse primers (**Fig 4.21**). There was a difference of 51 bp between the cattle (381 bp) and buffalo (330 bp) PCR products, which was sufficient to identify both the species when aligned with cattle and buffalo specific sequences.

In a similar study, using the common forward and species-specific reverse primers Matsunaga *et al.* (1999) differentiated 6 animal species (cattle, pig, chicken, sheep, goat and horse) by targeting mt cyt b gene. Also, Rodroguez *et al.* (2001) differentiated goose and duck by a multiplex PCR amplification of 5S rDNA gene.

In order to address the problems related to vetero-legal, quality-assurance and public health issues, the animal species identification comprising of cattle, buffalo, sheep, goat, pig and chicken would be inadequate. Therefore, a species-specific PCR have been developed in present study as an alternative approach for species identification. This consists of isolation of total DNA, PCR amplification of 12S rRNA gene, sequencing of the PCR product and its sequence analysis. Six species of non-food animals consisting of diverse groups of domestic (camel) as well as wild animals (leopard, muntjak and sika deer) along with a few species of birds (parakeet and kite) were also analyzed and compared with that of food animals.

4.5 Identification of animals by PCR-sequencing of mt 12S rRNA gene

PCR amplification of mt 12S rRNA gene of sika deer, leopard, muntjak, parakeet, black kite and camel using the universal primers yielded a product of 456 bp (Fig 4.22). These amplicons were sequenced using ABI prism and the sequences were submitted to the EMBL (European Molecular Biology Laboratory) database (Table 4.7). The corresponding sequences from other reports were retrieved from the GenBank (NCBI) and aligned using BLAST (www.ncbi.nlm.nih.gov/BLAST). Based upon the maximum score/alignment to the nearest species these animal species were identified and confirmed.

Later, the closely related species were aligned by '*Clustal*' method of *Megalign*® software (DNA STAR, inc. 1996) and compared with the most closely related species for similarity and divergence scores. Also, phylogenetic relationships between the related species were studied (Fig 4.23-4.28, Alignment 4.1-4.6).

4.5.1 BLAST analysis of mt 12S rRNA gene sequence for the identification of a species

In the present study, PCR amplification of mt 12S rRNA gene, its amplicon sequencing and sequence analysis was used as an alternative approach for the confirmation of 6 animal species i.e., parakeet, black kite, leopard, sika deer, muntjak and camel (Alignment 4.1-4.6 and Fig 4.22-4.28).

For the identification of animal species using sequence analysis, the sequence is introduced in to the distance matrix analysis and a tree is constructed with the sequences. The unknown sample is then clustered with the sequence of the reference species (available in database) to which the unknown sequence is phylogenetically most related (Quinteiro *et al.*, 1998).

Phylogeny and evolution of the antlered deer was determined from mitochondrial DNA (rRNA and tRNA genes) sequences (Miyamoto *et al.*, 1990). Using mitochondrial DNA sequences divergence, big cats and their hybrids were identified (Shankaranarayanan

and Singh, 1998). Amplification of mt 12S rRNA gene using universal primers, partial product sequencing and its analysis could help in the identification of an unknown species as mt 12S rRNA gene has been validated as the molecular marker in animal species identification by many other workers (Kocher *et al.*, 1989; Borgo *et al.*, 1996; Prakash *et al*. 2000 and Abdulmawjood and Buelte, 2001). However, apart from 12S rRNA gene, other mt genes, such as cyt b gene (Meyer *et al.*, 1995; Zimmerman *et al.*, 1998 and Verkaar *et al.*, 2002) and 16S rRNA gene (Borgo *et al*. 1996 and Abdulmawjood and Buelte, 2001), have also been targeted. Using the same approach Girish *et al.* (2004) identified cattle, buffalo, sheep, goat and mithun meat samples based on the sequence analysis of 12S rRNA gene, which is in accordance with the present study.

Differentiation of even closely related species was possible using this approach. As for as wild animals are concerned; leopard (*Panthera pardus*) was clearly differentiated from its similar species and other related felids such as lion (*Panthera leo*), lynx (*Lynx rufus*), puma (*Puma concolor*), cat (*Felis catus*), tiger (*Panthera tigris*), (Fig 4.25). Similarly, barking deer (Muntjak) and sika deer were also differentiated from the other closely related small ruminants and deer species (Fig 4.26 and 4.27). Besides, the avian species viz., black kite and parakeet were correctly identified and differentiated from the other closely avian species (Fig 4.23 and 4.24). The approach of PCR amplification of mt 12S rRNA gene and its sequence analysis could also be employed for the identification of any domestic animal species. In the present study, camel was identified and differentiated from its closely related species (Fig 4.28).

Briefly, there has been an unprecedented progress in the molecular biology; many techniques have been on the way of development of incorporating the enhanced sensitivity and quantitation precision so as to provide acceptable validity and reproducibility. Search for the detection methods with the better quantitative abilities should be continued. Incorporation

of these techniques for the purpose of meat identification is an easy task. Also, sincere efforts should be exercised by the researchers about the uncertainty associated with an analytical tool, and it should be sincerely provided along with the actual data and over-stating the accuracy may be stopped so that one can have a most reliable technique at their access to serve the mankind in a better way.

In order to prevent the fraud in the market and safeguard the interest of the consumers, the process of certification should become a mandate. It is necessary to validate a technique for the identification of the animal species. Principal consumer demand includes the quality animal products with confirmed origin and integrity of the components which the QA has to provide. The magnitude of the problem has gained momentum consequent to the introduction of BSE, which demand strict labeling norms especially beef in Europe (Commission Regulation EC No. 1825/2000). Fortunately, the Indian subcontinent is safe presently with respect to TSEs. However, the global liberal trade can not be ignored in the days to come. Hence, we need to be ready to address such catastrophes if at all they appear in future.

In the Indian context, animal species identification has really got social, religious, economic and political implications. In order to address such problems, the present piece of research work is an aid and its application would definitely benefit the scientific community to solve public health, QA, forensic, crime, insurance and wild animal related problems.

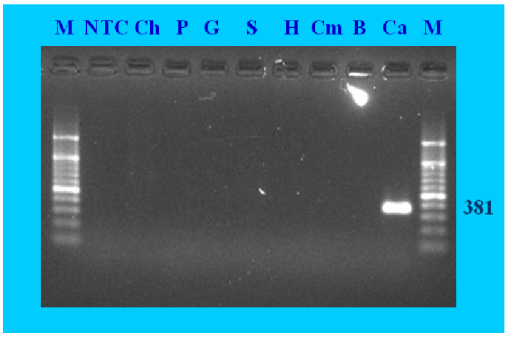


Fig 4.1 Cattle

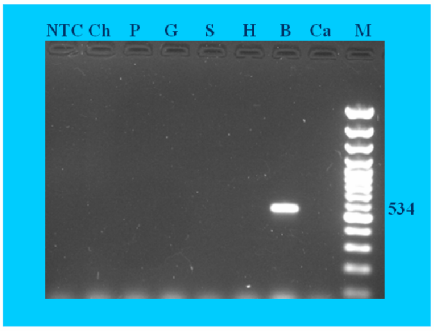


Fig 4.2 Buffalo

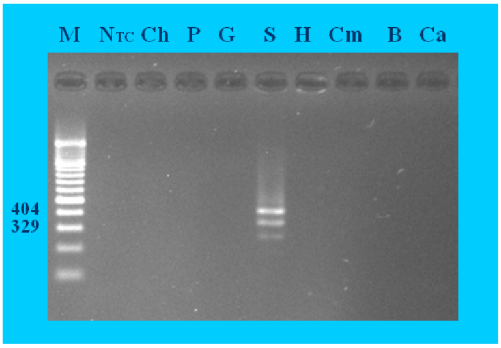


Fig 4.3 Sheep

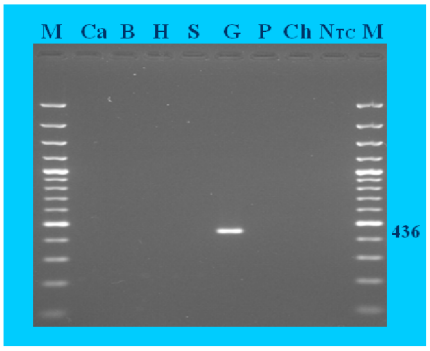


Fig 4.4 Goat

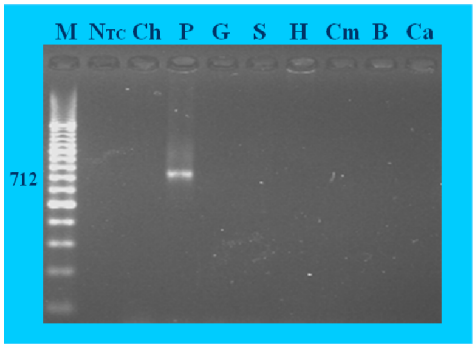


Fig 4.5 Pig

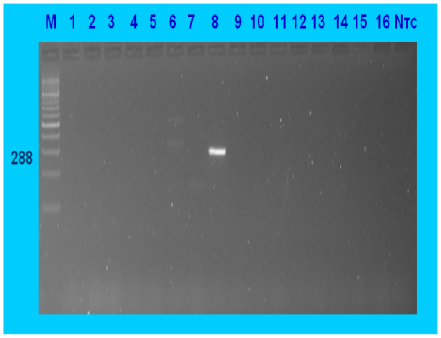


Fig 4.6 Chicken

Species-specific amplification of mt D-loop in cattle, buffalo, sheep, goat and pig and 5-amino-levulinate synthase gene of chicken

M-100bp marker, NTC-no template control, Ch-chicken, P-pig, G-goat, S-sheep, H-horse, Cm-camel, B-buffalo, Ca-cattle, Cm-camel, 1-Man, 2-Cattle, 3-Buffalo, 4-Sheep, 5-Goat, 6-Barking Deer, 7-Sika Deer, 8-Chicken, 9-Rabbit, 10-Horse, 11-Pig, 12-Leopard, 13-Royal Bengal Tiger, 14-Siberian tiger, 15-Dog, 16-Goral, 17-NTC

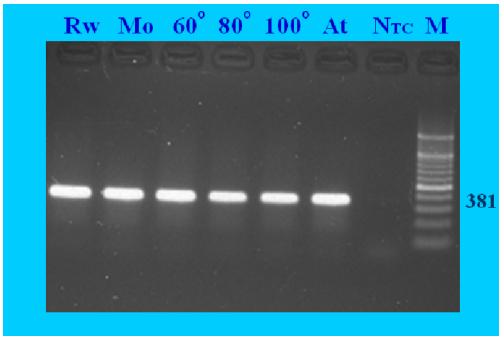


Fig 4.7 Cattle

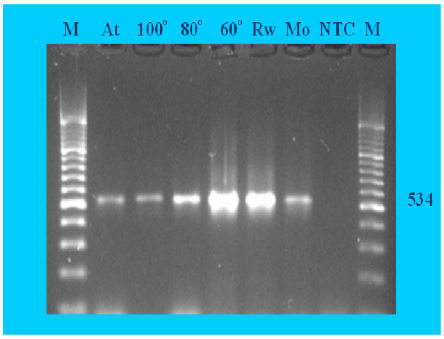


Fig 4.8 Buffalo

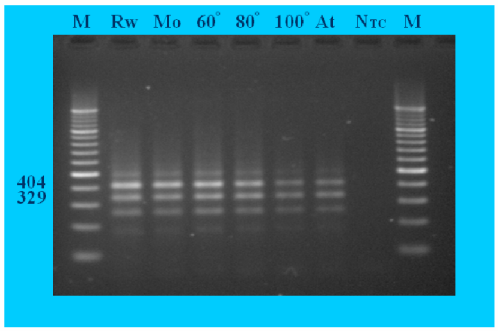


Fig 4.9 Sheep

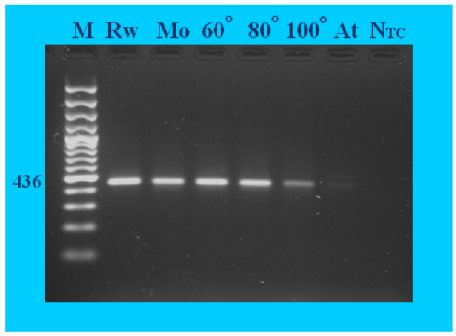


Fig 4.10 Goat

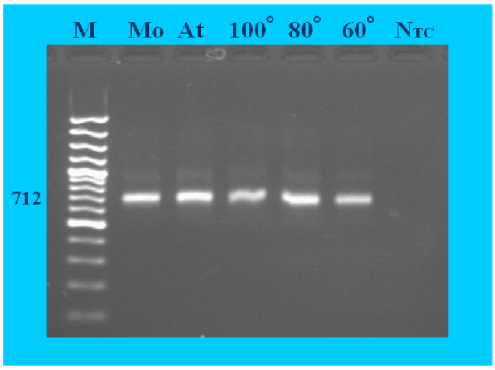


Fig 4.11 Pig

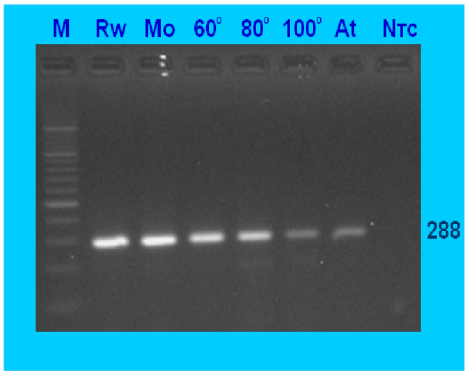


Fig 4.12 Chicken

Identification of cooked meat using species specific PCR assays

Rw-Raw; Mo-Micro-oven; At-autoclaved, NTC-no template control, M-100bp marker

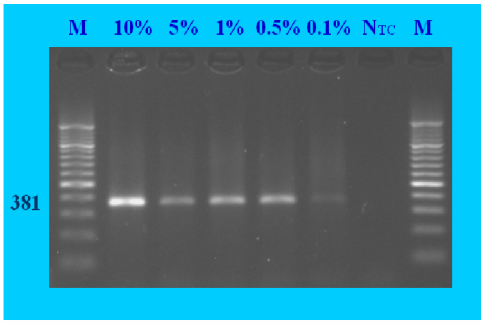


Fig 4.13 Cattle

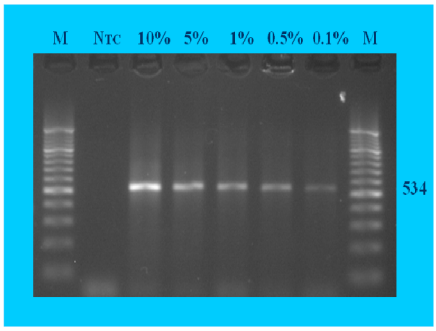


Fig 4.14 Buffalo

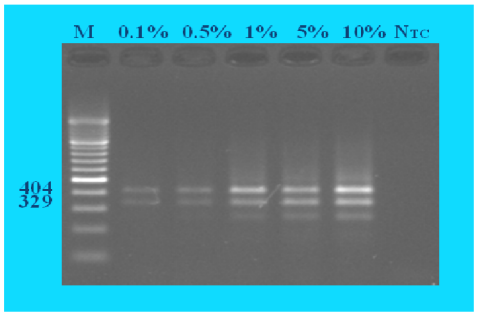


Fig 4.15 Sheep

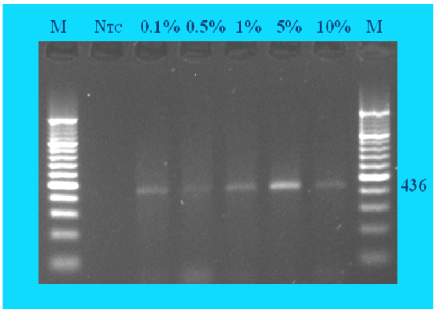


Fig 4.16 Goat

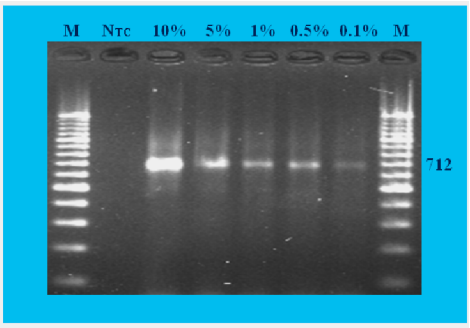


Fig 4.17 Pig

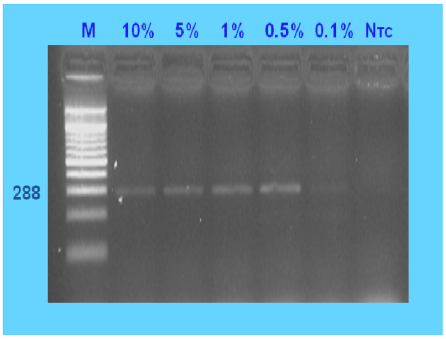


Fig 4.18 Chicken

Species-specific PCR for detection of specific species in the adulterated meat mixture

M-100bp marker; NTC-no template control

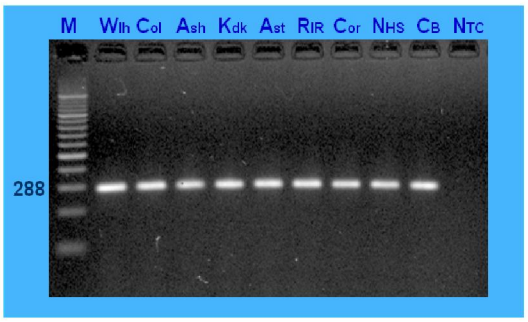


Fig 4.19 Chicken breeds

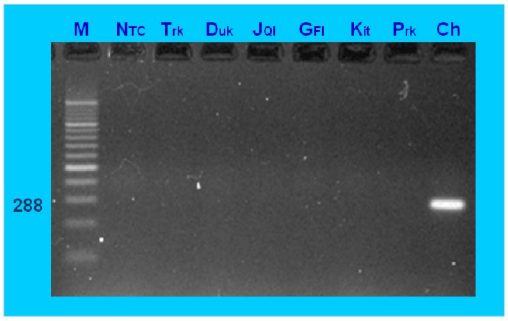


Fig 4.20 avian species

Chicken specific amplification of 5-aminolevulinate synthase gene
Lane M-100 bp Marker, 1-WLH, 2-Columbian, 3-Aseel, 4-Kadaknath, 5-Australorp, 6-Rhode Island Red, 7-Cornish (White), 8-New Hampshire, 9-Cross-bred (synthetic broiler), C-Negative Control

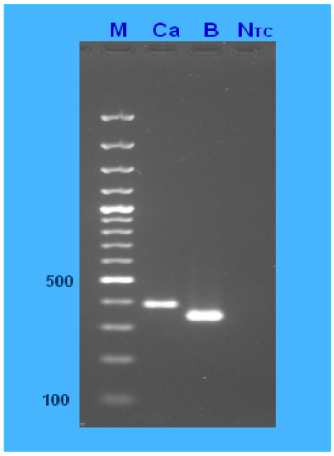


Fig 4.21 Multiplex PCR to differentiate cattle and buffalo (2% agarose gel) using species-specific forward and common reverse primers

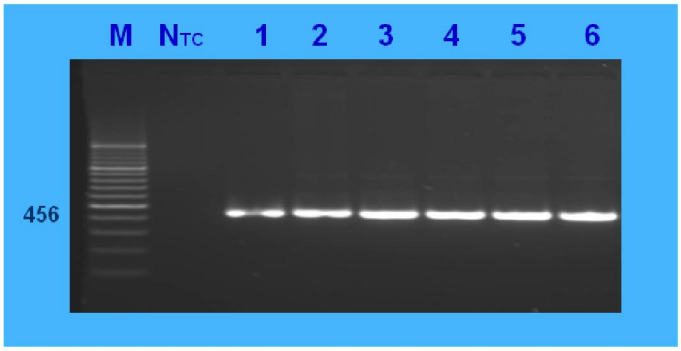


Fig 4.22 Amplification of mt 12S rRNA gene using universal primers
Sika deer, Leopard, Muntjak, Parakeet, Black Kite and Camel

Table 4.1 Recovery of total DNA (ng/mg) from the meat samples

Sample		Cattle	Buffalo	Sheep	Goat	Pork	Chicken
Raw meat		282-629	323-604.5	219-442	192.5-300	269.5-322.5	84.4-188
Thermally processed meat	60 ℃	274	205	334.4	297	46.4	84.4
	80 ℃	231	214.3	475.2	304	64.4	115
	100℃	298	227.2	523.2	315	113	168.6
	121 ℃	400	283.2	592.8	330	147.8	173.8
	Micro oven	452	350	407.2	351	297	205

Table 4.2 Details of the primer location, target, amplicon size and product length of species-specific primers

Species	Primers	Target	Target size	Primer locations		Amplicon Size	Location of amplicon
				Upper	Lower		
Cattle	VPH-CF & VPH-CR	mt D loop	911 bp	53-79	418-434	381 bp	53-434 (EF524180)
Buffalo	VPH-BF & VPH-BR	mt D loop	1,015 bp	168-192	682-701	534 bp	168-701 (AF475240)
Sheep	VPH-SF & VPH-SR	mt D loop	1,260 bp	148-164	453-476	329 bp	148-476 (DQ903304)
				148-164	528-551	404 bp	148-551 (DQ903233)
Goat	VPH-GF & VPH-GR	mt D loop	1,212 bp	98-121	513-533	436 bp	98-533 (DQ121616)
Pig	VPH-PF & VPH-PR	mt D loop	1,105 bp	558-579	1245-1269	712 bp	558-1269 (AM040615)
Chicken	VPH-ChF & VPH-ChR	5-aminolevulinate synthase exons 1-7	5,282 bp	6-29	272-293	288 bp	6-293 (X03517)

Table 4.3 Details of species-specific PCR conditions

Species	Primer Conc. (pico mol)		Annealing temp (°C)		Limit of detection (LOD)
	Range	Used	Range	Used	
Cattle	0.625-20	20	52-61.9	55	0.1 pg
Buffalo	0.625-20	20	45.1-55	55	10 pg
Sheep	0.25-20	20	50-59.9	55	1 pg
Goat	0.25-20	20	50-59.9	55	0.1 pg
Pig	0.25-20	20	46.1-55.8	55	10 pg
Chicken	1.25-20	20	53-62.9	55	10 pg

Table 4.4 Suitability of species-specific PCR assays in the thermally processed samples.

Species		Primers	Temperature				Micro-oven
			60°C	80°C	100°C	121°C	
1	Cattle	VPH-CF & CR	+	+	+	+	+
2	Buffalo	VPH-BF & BR	+	+	+	+	+
3	Sheep	VPH-SF & SR	+	+	+	+	+
4	Goat	VPH-GF & GR	+	+	+	+	+
5	Pig	VPH-PF & PR	+	+	+	+	+
6	Chicken	VPH-ChF & ChR	+	+	+	+	+

Table 4.5 Suitability of species-specific PCR assays for the detection of adulterated meat (n=20)

Species		Primers	Level of adulteration				
			10%	5%	1%	0.5%	0.1%
1	Cattle	VPH-CF & CR	+	+	+	+	+
2	Buffalo	VPH-BF & BR	+	+	+	+	+
3	Sheep	VPH-SF & SR	+	+	+	+	+
4	Goat	VPH-GF & GR	+	+	+	+	+
5	Pig	VPH-PF & PR	+	+	+	+	+
6	Chicken	VPH-ChF & ChR	+	+	+	+	+

Table 4.6 Statistical analysis of species-specific PCR assays involving phenotype vs. PCR identification of animal species in an intra-laboratory validation study[Ŧ]

Species	Primers	Inclusivity * (%)	Exclusivity** (%)	Positive predictivity (%)	Negative predictivity (%)	Analytical accuracy (%)	Kappa index
Cattle	VPH-CF & CR	100	100	100	100	100	1
Buffalo	VPH-BF & BR	100	100	100	100	100	1
Sheep	VPH-SF & SR	100	100	100	100	100	1
Goat	VPH-GF & GR	100	100	100	100	100	1
Pig	VPH-PF & PR	100	100	100	100	100	1
Chicken	VPH-ChF & ChR	100	100	100	100	100	1

***** Inclusively was assessed using the available breeds and cross-bred
****** Exclusivity was assessed in 25 animal species (including breeds/ strains)
Ŧ Sample size (n) = 20 raw meat samples

Table 4.7 Sequences of 12S rRNA gene submitted to GenBank

	Species	Scientific name	Target	Accession number
1	Sika deer	*Cervus nippon*	12S rRNA gene	AM849037
2	Leopard	*Panthera pardus*	12S rRNA gene	AM779888
3	Muntjak	*Muntiacus muntjak*	12S rRNA gene	AM778453
4	Parakeet	*Psittacula krameri*	12S rRNA gene	AM778108
5	Black Kite	*Milvus migrans*	12S rRNA gene	AM778107
6	Camel	*Camelus dromedarus*	12S rRNA gene	AM936925
7	Goral	*Naemorhedus goral*	12S rRNA gene	FM179469
8	Bengal tiger	*Panthera tigris tigris*	12S rRNA gene	FM179470
9	Siberian tiger	*Panthera tigris altaica*	12S rRNA gene	FM179471

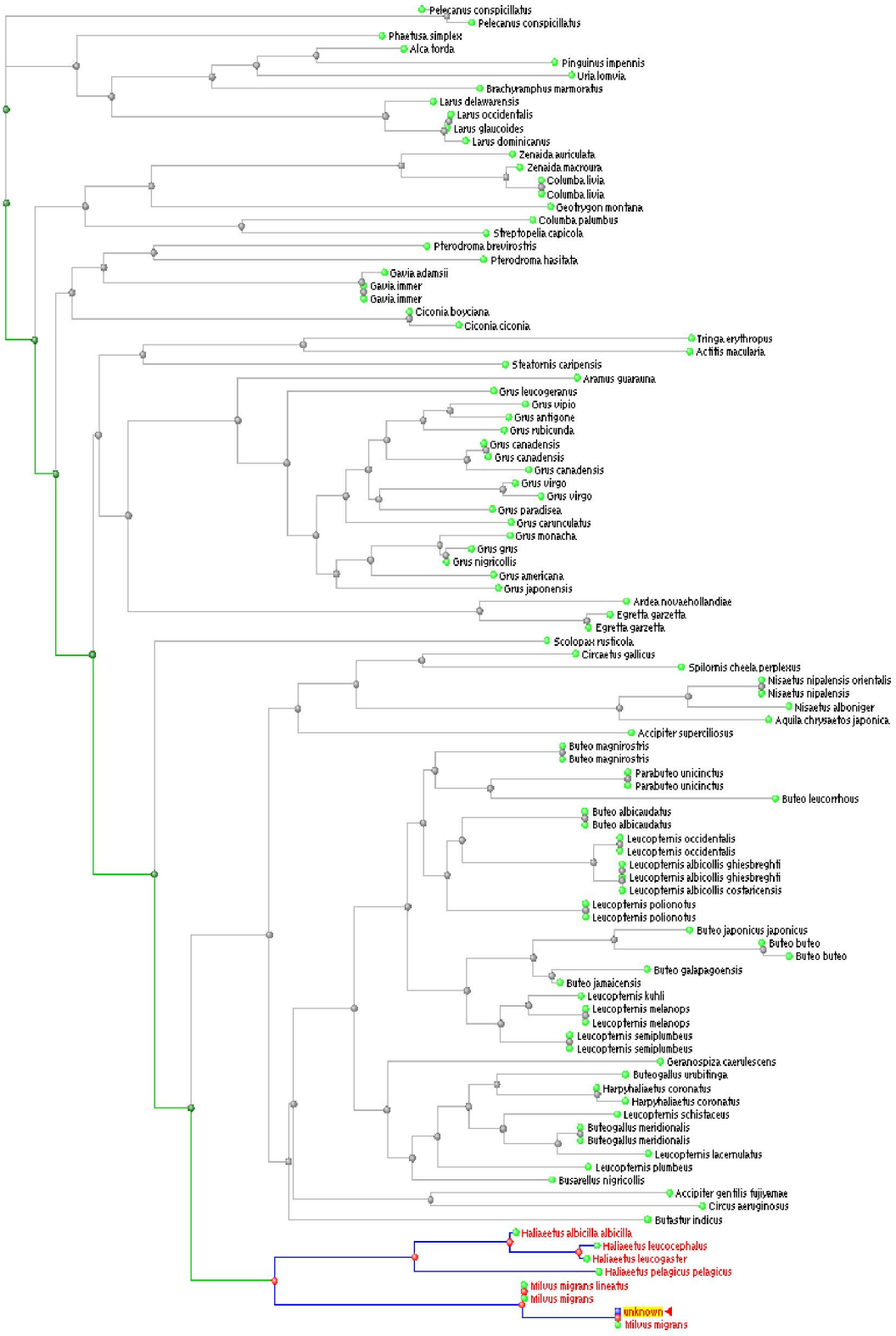


Fig 4.24 Distance tree of black kite (*Milvus migrans*) mt 12S rRNA gene sequence produced using BLAST pairwise alignments

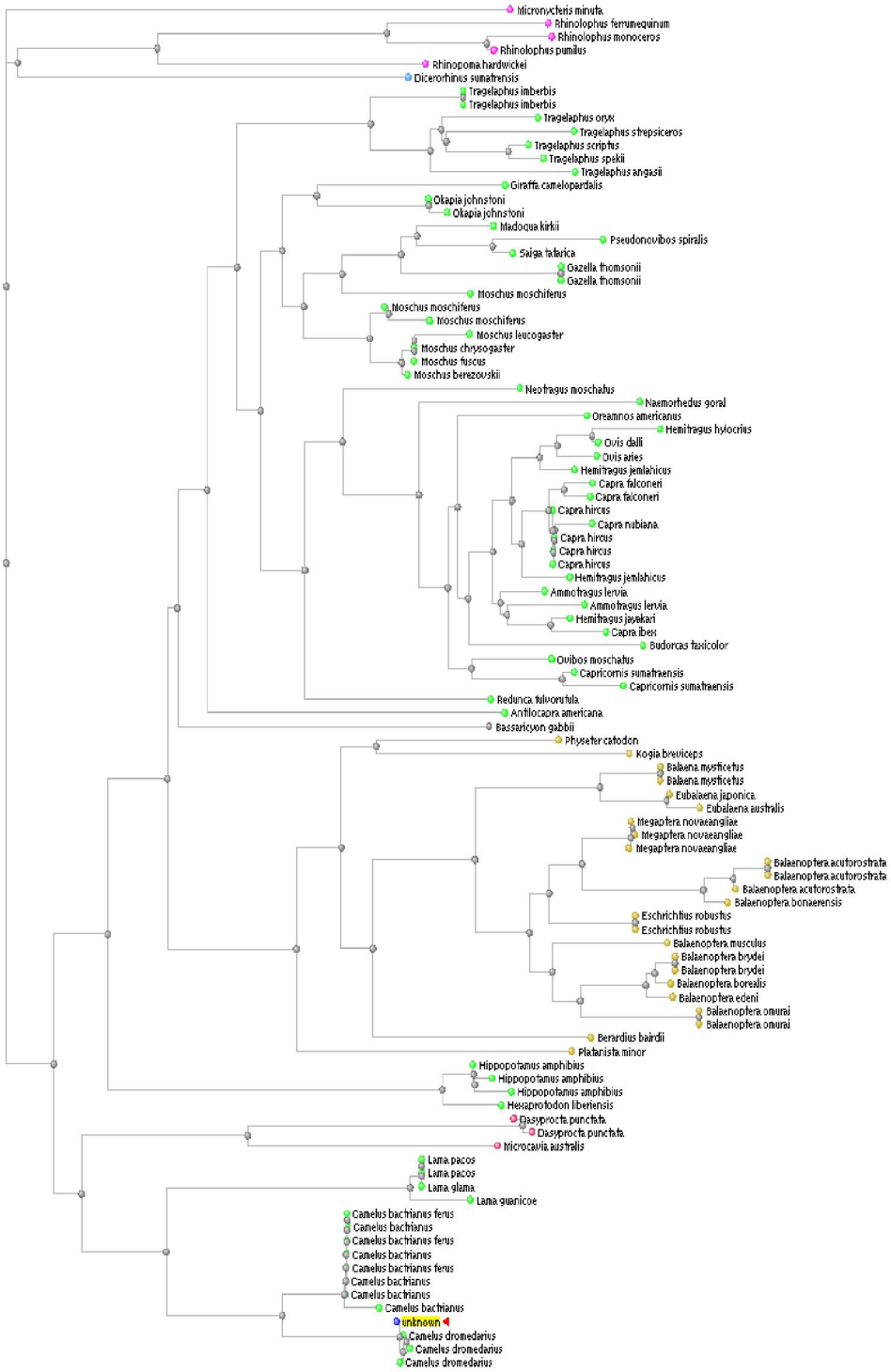


Fig 4.28 Distance tree of camel (*Camelus dromedaries*) mt 12S rRNA gene sequence produced using BLAST pairwise alignments

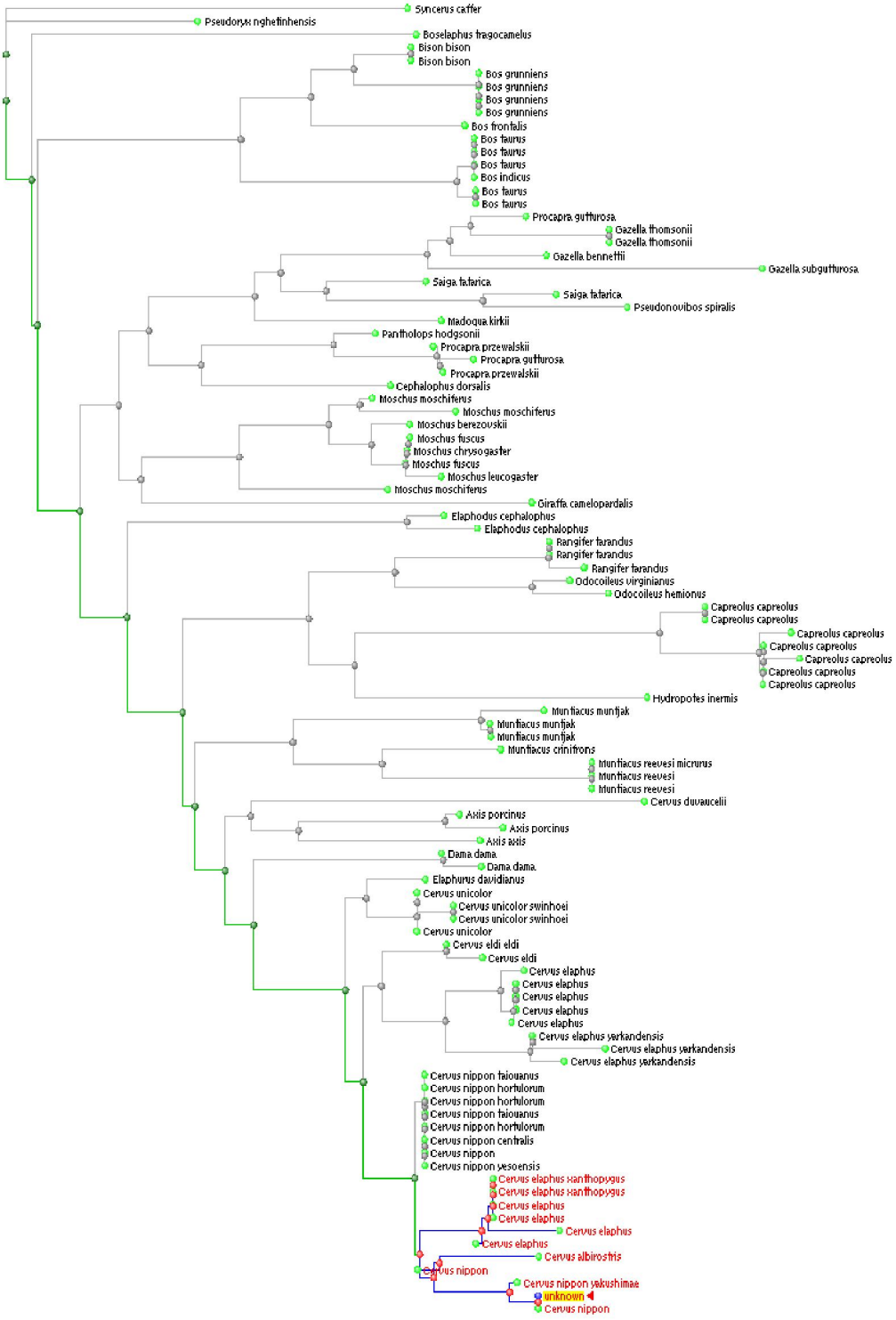


Fig 4.26 Distance tree of sika deer (*Cervus nippon*) mt 12S rRNA gene sequence produced using BLAST pairwise alignments

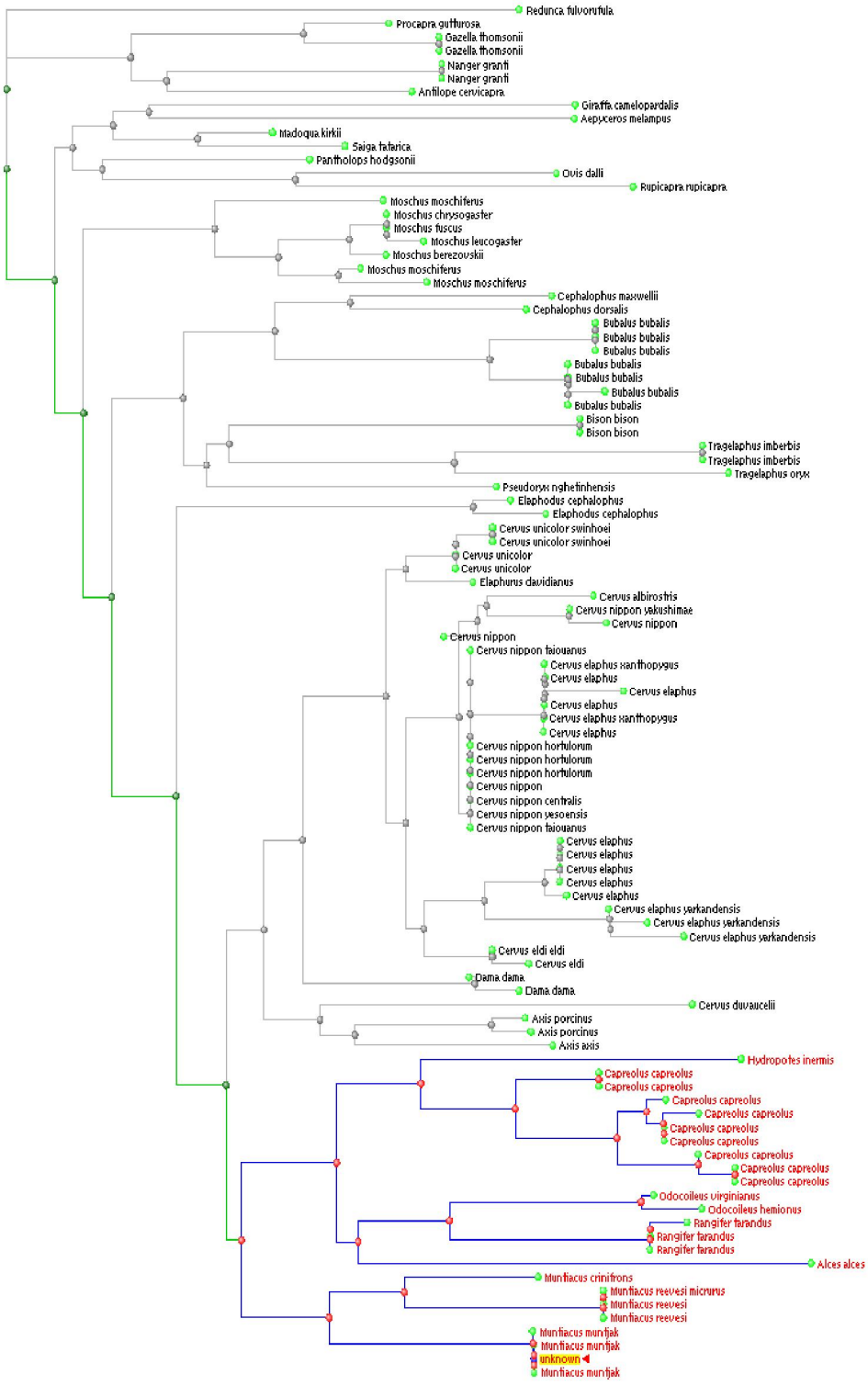


Fig 4.27 Distance tree of Indian muntjak (*Muntiacus muntjak*) mt 12S rRNA gene sequence produced using BLAST pairwise alignments

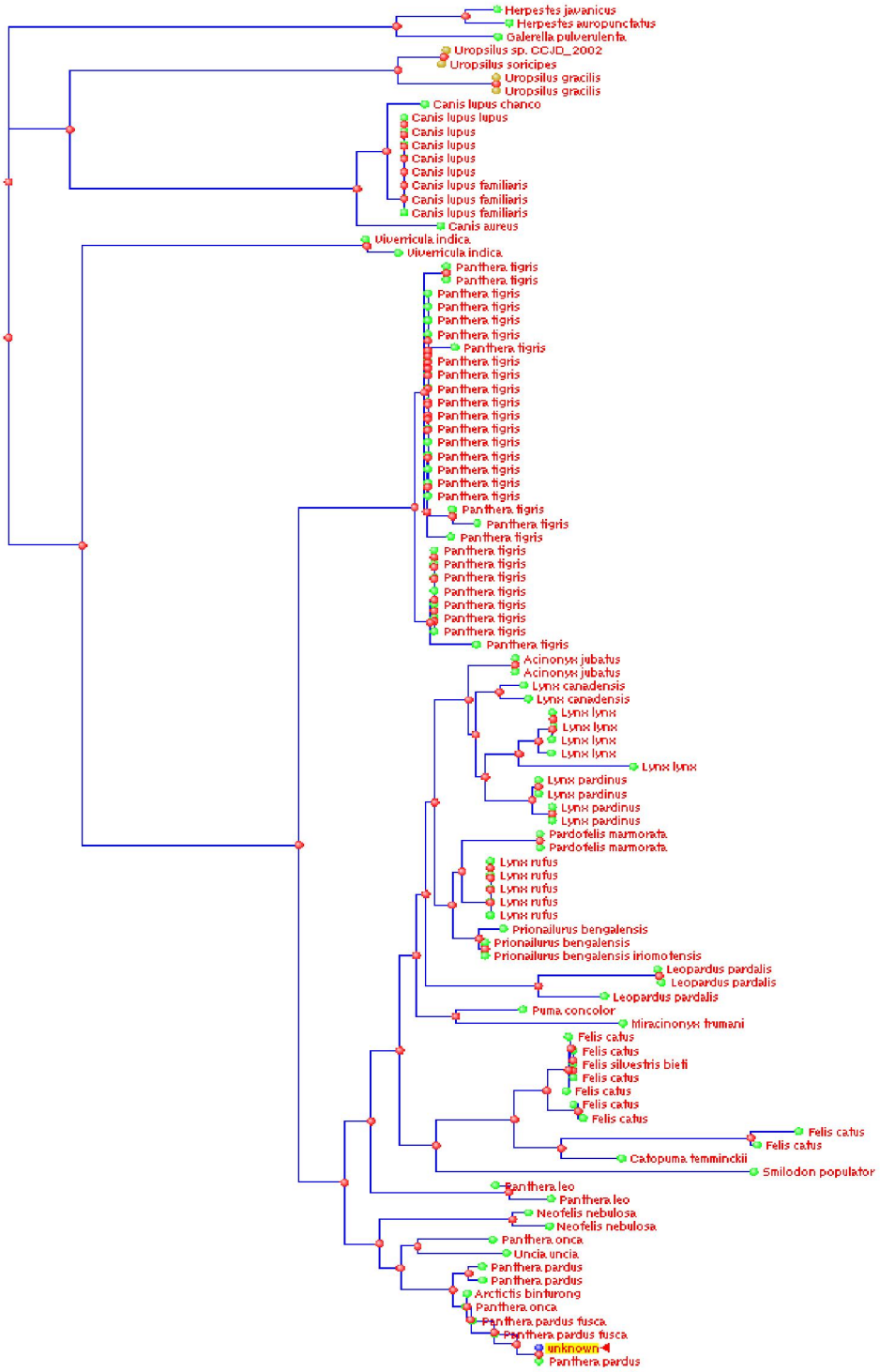


Fig 4.25 Distance tree of leopard (*Panthera pardus*) mt 12S rRNA gene sequence produced using BLAST pairwise alignments

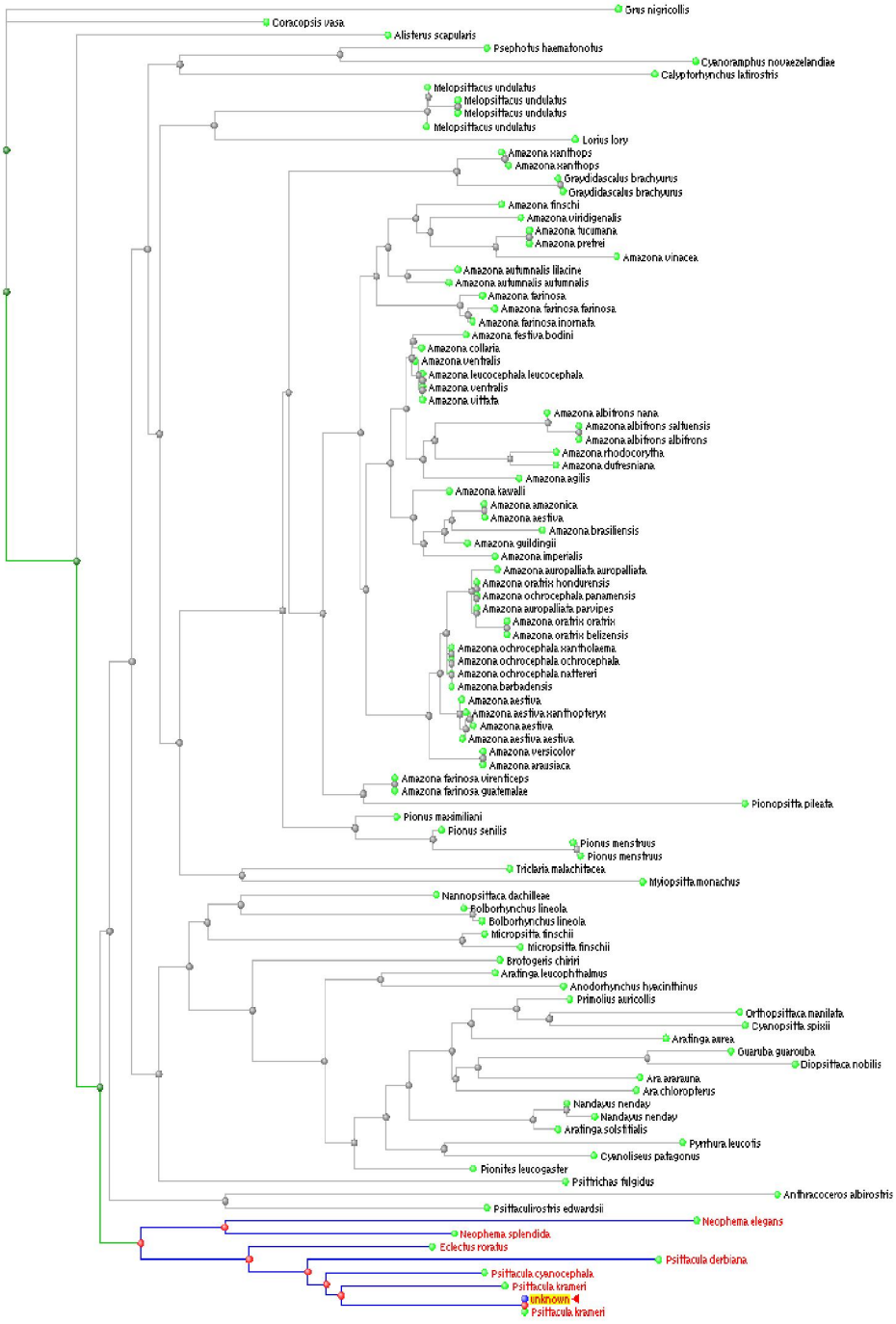
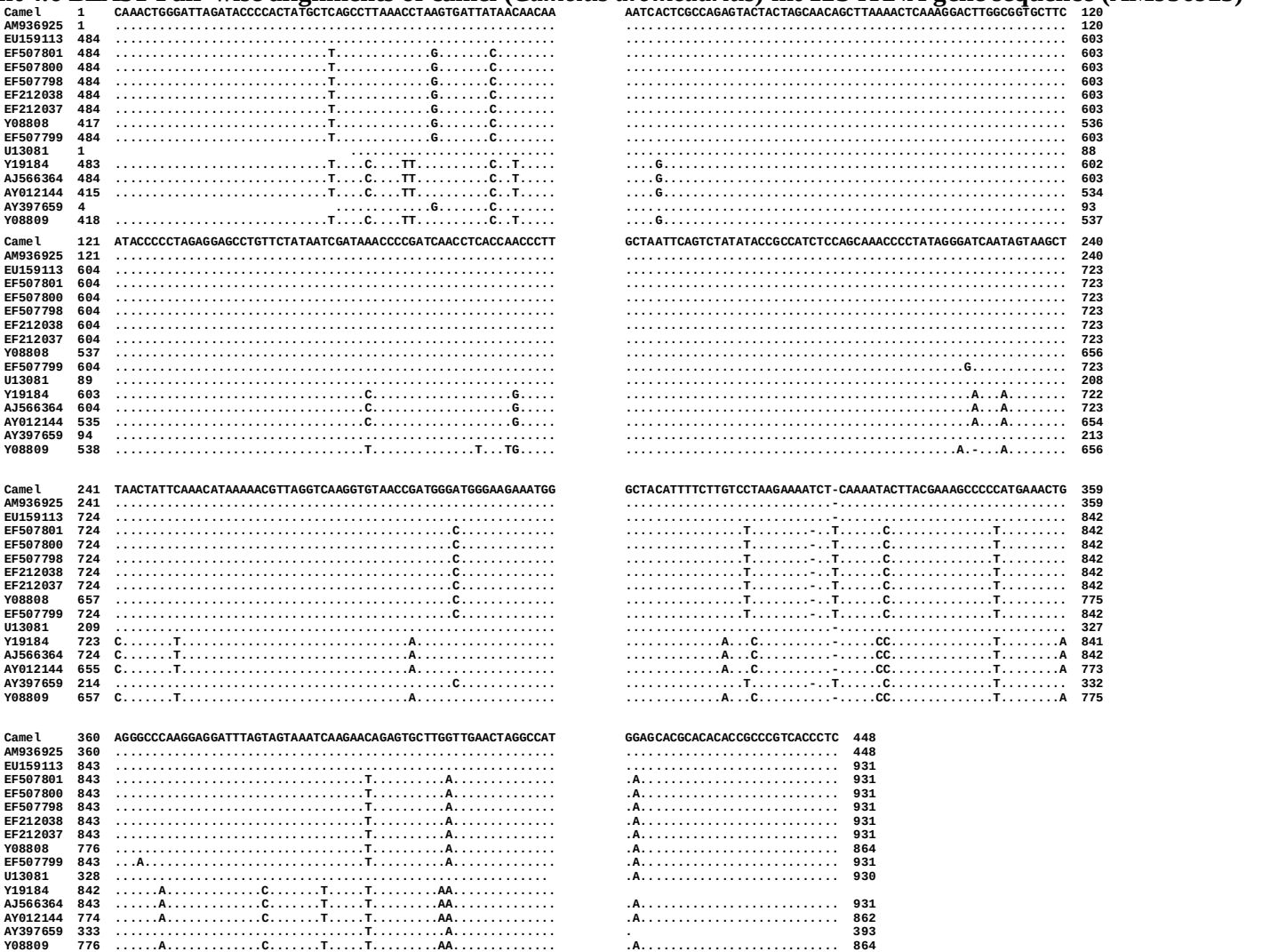


Fig 4.23 Distance tree of parakeet (*Psittacula krameri*) mt 12S rRNA gene sequence produced using BLAST pairwise alignments

Alignment 4.2 BLAST (Pair-wise alignments) of black kite (*Milvus migrans*) mt 12S rRNA gene sequence (AM778108)

```
M.migrans 1    GACCTGGCCCT-AATCTTG-CCCTTATCCTACCAAGGTATCCGCCTGAGAACTACGAGCA  58
AM778107  1    ...........-.......-..........................................  58
AB219546  460    .........A.......ATA.....................................  517
U83721    460    .........A.......ATA.....................................  517
AB219552  462    .........A.......ATA....C..C.....A.......................  519
AB219553  461    .........A.......ATA....C..C.....A.......................  518
U83722    460    .........A.......ATA....C..C.....A.......................  517
U88011    2      .........A.......ATA....C..C.....A.......................  59

M.migrans 119  TACAATCGATAACCCACGCTACACCTCACCACTTCTTGCCCACAGCAGCCTACATACCGC  178
AM778107  119  ............................................................  178
AB219546  578  ............................................................  637
U83721    578  ............................................................  637
AB219552  580  ..T..............G....C.............A..T.................  639
AB219553  579  ..T..............G.....................T.A.................  638
U83722    578  ..T..............G....C.............A..T.................  637
U88011    120  ..T..............G....C.............A..T.................  179

M.migrans 238  AAAAGACAGGTCAAGGTATAGCTCACGAAGTGGAAGAAATGGGCTACATTTTCTA-AGAT  296
AM778107  238  ............................................................  296
AB219546  697  ............................................................  755
U83721    697  ............................................................  755
AB219552  698  ..........................................G..-.............  756
AB219553  698  G.........................................-.A..............  756
U83722    696  ..........................................G..-.............  754
U88011    238  ..........................................G..-.............  296

M.migrans 357  GAGACAATCGAGCTCTATTTAAACTGGCCCTGGAGCACGTACACACCGCCACCATCACCC  416
AM778107  357  ............................................................  416
AB219546  816  ..........................................T......-.G-......  873
U83721    816  ..........................................T......-.G-......  873
AB219552  817  .G......A....C........G..........G.........T......-.G-......  874
AB219553  817  .G......AA...C..........C.........G.........T......-.G-......  874
U83722    815  .G......G....C..G.....G..........G.........T......-.G-......  872
U88011    357  .G......G....C..G.....G..........                            389

M.migrans 59   CAAACGCTTGAAACTCTAAGGACTTGGCGGTGCCCCAAACCCACCTAGAGGAGCCTGTTC  118
AM778107  59   ............................................................  118
AB219546  518  ............................................................  577
U83721    518  ............................................................  577
AB219552  520  .........A..................................................  579
AB219553  519  .........A..................................................  578
U83722    518  .........A..................................................  577
U88011    60   .........A..................................................  119

M.migrans 179  CGTCGCCAGCTCACCTCTCCTGAGAG-TTCAACAGTGAGCACAATAGCTTCTCCCCGCTA  237
AM778107  179  ........................-...................................  237
AB219546  638  ........................-...................................  696
U83721    638  ........................-...................................  696
AB219552  640  ..................T.......C..-.G...................-........  697
AB219553  639  .....T............T.......C..-.GT..................C........  697
U83722    638  ..................T.......C..-.G........T.........-........  695
U88011    180  ..................T.......C..-.G...................-........  237

M.migrans 297  AGAAAACCACACGGAAGGGGACATGAAACAGCCCCCGGAAGGCGGATTTAGCAGTAAACT  356
AM778107  297  ............................................................  356
AB219546  756  ............................................................  815
U83721    756  ............................................................  815
AB219552  757  .......T...................TG...............................  816
AB219553  757  .......T...................T................................  816
U83722    755  .......T.............G.......TG.............................  814
U88011    297  .......T............G.......TG.............................  356

M.migrans 417  TCATG  421
AM778107  417  .....  421
AB219546  874  ..     875
U83721    874  ..     875
AB219552  875  ..     876
AB219553  875  ..     876
U83722    873  ..     874
```

Alignment 4.6 BLAST Pair-wise alignments of camel (*Camelus dromedarius*) mt 12S rRNA gene sequence (AM936925)



Alignment 4.4 BLAST (Pair-wise alignments) of sika deer (*Cervus nippon*) mt 12S rRNA gene sequence (AM849037)

```
C. nippon 1    GCCGGATGCCTAGCCTT-ACCAC-AATAGTTATATAAACAAAACTATTCGCCAGAGTACT  58
AM849037  1    .................-.....-.................................... 58
AB218689  505  ...........A.A...A.................................... 559
DQ191148  436  ...........A.A...A.........G.......G...G.............. 490
DQ191146  436  ...........A.A...A.........G.......G...G.............. 490
AY184431  436  ...........A.A...A.........G.......G...G.............. 490
AY184430  436  ...........A.A...A.........G.......G...G.............. 490
AY184429  436  ...........A.A...A........C.......................... 490
DQ191152  1                                  ......................... 26
DQ191153  1                                  .......G...G.............. 26
DQ191154  1                                  .......G...G.............. 26
C. nippon 119  TGTTCTATAATCGATAAACCCCGATAAACCTCACCATTCCTTGCTACTACAGTCTATATA 178
AM849037  119  .......................................................... 178
AB218689  620  .......................................................... 679
DQ191148  551  .......................................................... 610
DQ191146  551  .......................................................... 610
AY184431  551  .......................................................... 610
AY184430  551  .......................................................... 610
AY184429  551  ......................................C................... 610
DQ191152  87   .......................................................... 146
DQ191153  87   .......................................................... 146
DQ191154  87   .......................................................... 146
C. nippon 239  ACGTTAGGTCAAGGTGTAACCTATGGAACGGAAAAAAATGGGCTACATTTTCTAATCTAA 298
AM849037  239  .......................................................... 298
AB218689  740  .......................................................... 799
DQ191148  671  ................................G......................... 730
DQ191146  671  ................................G......................... 730
AY184431  671  ................................G......................... 730
AY184430  671  ................................G......................... 730
AY184429  671  ................................G......................... 730
DQ191152  207  ................................G......................... 266
DQ191153  207  ................................G......................... 266
DQ191154  207  ................................G......................... 266
C. nippon 358  CTAAGAATAGAGTGCTTAGTTGAATTAGGCCATGAAGCACGCACACCCCCC  408
AM849037  358  ..........................................................  408
AB218689  859  ..........................................                904
DQ191148  790  ..........................................                835
DQ191146  790  ..........................................                835
AY184431  790  ..........................................                835
AY184430  790  ..........................................                835
AY184429  790  ..........................................                835
DQ191152  326  ..........................................                371
DQ191153  326  ..........................................                371
DQ191154  326  ........................T.................                371
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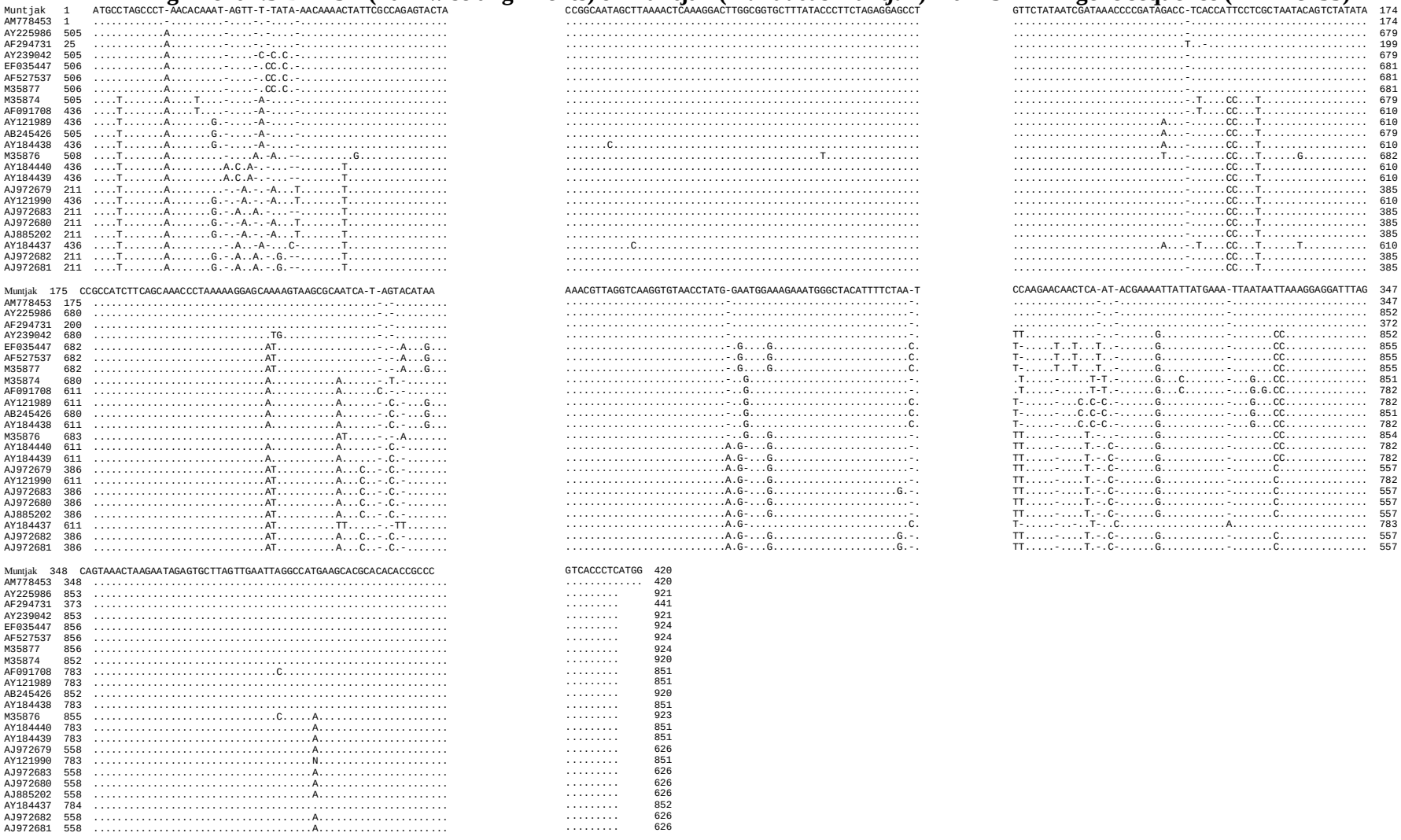
```
C. nippon 59   ACCGGCAATAGCTTAAAACTCAAAGGACTTGGCGGTGCTTTATACCCTTCTAGAGGAGCC  118
AM849037  59   ..........................................................  118
AB218689  560  ..........................................................  619
DQ191148  491  ..........................................................  550
DQ191146  491  ..........................................................  550
AY184431  491  ..........................................................  550
AY184430  491  ..........................................................  550
AY184429  491  .....................................C....................  550
DQ191152  27   ..........................................................   86
DQ191153  27   ..........................................................   86
DQ191154  27   ..........................................................   86
C. nippon 179  CCGCCATCTTCAGCAAACCCTAAAAAGGTACAAAAGTAAGCACAATCATAATACATAAAA  238
AM849037  179  ..........................................................  238
AB218689  680  ..........................................................  739
DQ191148  611  ..........................................................  670
DQ191146  611  ..........................................................  670
AY184431  611  ..........................................................  670
AY184430  611  ..........................................................  670
AY184429  611  ..........................................................  670
DQ191152  147  ..........................................................  206
DQ191153  147  ..........................................................  206
DQ191154  147  ..........................................................  206
C. nippon 299  GAAAA-TCCAACACGAAAGTTATTATGAAACTAGTAACCAAAGGAGGATTTAGCAGTAAA  357
AM849037  299  .....-....................................................  357
AB218689  800  .....-....................................................  858
DQ191148  731  .....-.........................A..........................  789
DQ191146  731  .....-.........................A..........................  789
AY184431  731  .....-.........................A..........................  789
AY184430  731  .....-.........................A..........................  789
AY184429  731  .....C..-.....................A..........................  789
DQ191152  267  .....-.........................A..........................  325
DQ191153  267  .....-.........................A..........................  325
DQ191154  267  .....-.........................A.....................C....  325
```

Alignment 4.5 BLAST (Pair-wise alignments) of muntjak (*Mantiacus muntjak*) mt 12S rRNA gene sequence (AM778453)



Alignment 4.3 BLAST (Pair-wise alignments) of leopard (*Panthera pardus*) mt 12S rRNA gene sequence (AM849037)

```
Leopard   1     GATCCTTGGCCCT-AACCTAGATAGTTAGC-CC-ACCCCAACTATCCGCCAGAGAACTAC  57
AM779888  1     .............-..............-..-.........................  57
AF416456  5           .....-..............-..A.A.AA..................-.  53
AY012151  444       ...A.....A.............T.A-.A.AA.....................  498
DQ257669  1411      ...A.....A.............T.G-.A.AA.....................  1465
DQ286781  371       ...A.....A..............-..A.A.AA.....................  317
D28895    2         ...A.....A..............-..A.A.AA.....................  56
D28896    2         ...A.....A..............-..A.A.AA.....................  56
EF199741
EF203401
D28897    2         ...A.....A.............T.A-.A.AA.....................  56
AY499283  2         ...A.....A.............T.G-.-.AA.....................  55

Leopard   117   GTTCTATAATCGATAAACCCCGATAAACCTCACCATCTCTTGTTAATTCAGCCTATATAC  176
AM779888  117   ............................................................  176
AF416456  113   ............................................................  172
AY012151  558   .....................................C......................  617
DQ257669  1526  ......................G.............CC.....................  1585
DQ286781  257   ............................................................  198
D28895    116   ............................................................  175
D28896    116   .....................................C......................  175
EF199741  53    ............................................................  112
EF203401  53    ............................................................  112
D28897    116   .....................................C......................  175
AY499283  115   .....................................CC.....................  174

Leopard   237   AAGTTAGGTCAAGGTGTAGCCTATGAGATGGGAAGCAATGGGCTACATTTTCTACAACTA  296
AM779888  237   ............................................................  296
AF416456  233   ............................................................  292
AY012151  678   ..................C.........................................  737
DQ257669  1646  ..................C.........................................  1705
DQ286781  137   ............................................................  78
D28895    236   ............................................................  295
D28896    236   ............................................................  295
EF199741  173   ............................................................  232
EF203401  173   ............................................................  232
D28897    236   ..................C.........................................  295
AY499283  235   ....-.............C.........................................  293

Leopard   357   AGAATAGAGAGCTCAATTGAATCGGGCCATGAAGCACGCACACAC-GCCCGAGCTCACAC  415
AM779888  357   .............................................-..............  415
AF416456  353   .........................................................C.....  403
AY012151  798   ..................T......................C.....  848
DQ257669  1766  ...G.....................................C.....  1816
DQ286781  17    .................  1
D28895    356   .................  373
D28896    356   .................  373
EF199741  293   .............................................C....  342
EF203401  293   .............................................C....  342
D28897    356   ............T....  373
AY499283  354   ...G........................  382

17262     416   TACATGGGGGGGGGGGGGGGAAAAGGGGGGGTCTTAGAGTTCAAGCGGAGCAGAGTTCA  475
AM779888  416   ............................................................  475

Leopard   58    TAGCAACAGCTTAAAACTCAAA-GGACTTGGCGGTGCTTTATATCCCTCTAGAGGAGCCT  116
AM779888  58    ......................-.....................................  116
AF416456  54    ......................-.....................................  112
AY012151  499   ......................-.....................................  557
DQ257669  1466  ......................G.....................................  1525
DQ286781  316   ......................-.....................................  258
D28895    57    ......................-.....................................  115
D28896    57    ......................-.....................................  115
EF199741  1           ...............-.....................................  52
EF203401  1           ...............-.....................................  52
D28897    57    ......................-.....................................  115
AY499283  56    ......................-...........N.........................  114

Leopard   177   CGCCATCTTCAGCAAACCCTAAAAAGGAAGAAAAGTAAGCACAAGTGTCTTAACACAAAA  236
AM779888  177   ............................................................  236
AF416456  173   ............................................................  232
AY012151  618   .............................................A....G........  677
DQ257669  1586  ............................................................  1645
DQ286781  197   ............................................................  138
D28895    176   .............................................T..............  235
D28896    176   .............................................T..............  235
EF199741  113   ............................................................  172
EF203401  113   ............................................................  172
D28897    176   ..............................G..............G.....  235
AY499283  175   ............................................................  234

Leopard   297   GAACATCCACGAAAATCCTTATGAAATTAAGTATTCAAGGAGGATTTAGTAGTAAATTTG  356
AM779888  297   ............................................................  356
AF416456  293   ............................................................  352
AY012151  738   ............................A...............................  797
DQ257669  1706  ...T.C.........................A.........................  1765
DQ286781  77    ............................................................  18
D28895    296   ............................................................  355
D28896    296   ............................................................  355
EF199741  233   ............................................................  292
EF203401  233   ............................................................  292
D28897    296   ............................................................  355
AY499283  294   ...T.C.........................A.........................  353

Leopard   476   GAGGGAA  482
AM779888  476   .......  482
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Alignment 4.1 BLAST (Pair-wise alignments) of parakeet (*Psittacula krameri*) mitochondrial 12S rRNA gene sequence (AM778108)

```
P.krameri 1    ACGCCTGGCCCT--ATCCCGATG-T-TCCTAGCACTTAAAACATCCGCCCGAGAACTACG  56
AM778108  1    ............--.........-.-.....................................  56
EU197058  1          ......AA.........-.-...C.A...A.........................  52
EU197080  3       ....A.....AA...T......-.C...-.A...A...G...................  58
EU197074  1                                                ...................  19
U88021    1       ..........AA.........T.-...-.A...A.........................  56
AY274018  522                                          .................T....  543
EU197067  4                                                .............T..  19

P.krameri 57   AGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCCCTAAACCCACCTAGAGGAGCCT  116
AM778108  57   ............................................................  116
EU197058  53   ............................................................  112
EU197080  59   ............................................................  118
EU197074  20   ............................................................  79
U88021    57   ............................................................  116
AY274018  544  ............................................................  603
EU197067  20   ...C........................................................  79

P.krameri 117  GTTCTATAATCGATAACCCACGATACACCCAACCACTTCTTGCCAAAACAGCCTACATAC  176
AM778108  117  ............................................................  176
EU197058  113  ...........................................G................  172
EU197080  119  ............................................................  178
EU197074  80   ............................................................  139
U88021    117  ..........................G.................................  176
AY274018  604  .....G..............T.......C.........A...................  663
EU197067  80   ....................................A....C..............  139

P.krameri 177  CGCCGTCAT-AGCCCACCTCCA-TGAGAGCATAATAGCGAGCCCAATAGCCCACC--C--  230
AM778108  177  .........-...........-.....................................--.--  230
EU197058  173  .........-...........-........C.GC................T.T.AA.--  228
EU197080  179  ........C-..........T.-.........C..C...........C........AA.--  234
EU197074  140  ........C-..........T..-...A....C..C........A.......-...AA.--  194
U88021    177  ........C-.....G...T..-...A....C.GC.................G..--.--  230
AY274018  664  .......G.C..........A.-......A....C..T........G.....TT.--AG-  719
EU197067  140  ........GC..........-.G......A.C..C..T........G....-...--.AG  195

P.krameri 231  CCCGCTAACAAGACAGGTCAAGGTATAGCCCACGAAGTGGAAGAAATGGGCTACATTTCC  290
AM778108  231  ............................................................  290
EU197058  229  ............................................................  288
EU197080  235  .......GT....................................................  294
EU197074  195  ............................T...............................  254
U88021    231  ..-.........................................................  289
AY274018  720  ...A....A.....................................................T.  779
EU197067  196  .....................................................T.  255

P.krameri 291  TAA-GATA-G-AA-A-A-CC--A-CGAAAAGGGGCCTGAAACCTGCCCCTAAAAGGCGGA  341
AM778108  291  ...-....-.-..-.-.-..--.-.....................................  341
EU197058  289  ...-A...A.-..-.-.-..--.-...........................G........  340
EU197080  295  ...-A...-.T..-.-.-..C-.A...................A......G........  348
EU197074  255  ...-A...-.-..-.-.-..C-.-.....G.................C.G........  306
U88021    290  ...-A...-.-G.-C-.-..CC.-........A..T.......T.A......G........  342
AY274018  780  ...-A...-.-..C.-.A..---.A..G.......TA........A......G........  833
EU197067  256  ...A.-..-.-..C.T.-..--.A..........TA........A......G........  309

P.krameri 342  TTTAGCAGTAAAGA-GAG--ACAATCATGCTCTCCTTAAGCCGGTCCTAGGGCACGTACA  398
AM778108  342  ............-...--.........................................  398
EU197058  341  ............-...--............T...................          391
EU197080  349  ...........-..A...--...................AA...................  405
EU197074  307  ............-...--.....................................      357
U88021    343  ............-...--.T.................A.............          392
AY274018  834  ..........G..-...GG.......A..C.C......A...................  892
EU197067  310  ............-.G.--..........C.............................  366

P.krameri 399  CACCGCCCCGTCACCCTCAA  418
AM778108  399  ....................  418
AY274018  893  ........-.........     909
```

5 Summary & Conclusion

Identification of meat of different species of food animals is considered important because of religious, social, forensic and public health reasons. In order to detect extent of adulteration in meat and animal derived products, various methodologies based on physical, chemical, electrophoretic, immunological and chromatographic principles have been developed. These conventional techniques suffer from one or the other limitation with respect to sensitivity, specificity, repeatability and reproducibility. At the present time, DNA based molecular techniques particularly PCR and its variants are the most preferred one for identification of animal species because of their specificity, sensitivity, accuracy and precision.

In view of the above, the present study was designed with the objective to develop species-specific PCR assays for the identification of cattle, buffalo, sheep, goat, pig and chicken in raw, adulterated as well as thermally processed samples. Further a total of 25 animal species comprising of domestic, wild and pet animals as well as some avian and fish species were also analyzed employing these techniques. The total DNA was isolated using either Wizard genomic purification kit or by phenol-chloroform method from the tissues (raw, adulterated and processed), blood and semen. The isolated DNA was checked for purity, quality and concentration and later used for PCR amplification.

Species-specific PCR assays were developed for cattle, buffalo, sheep, goat and pig against the specific region of mitochondrial (mt D-loop); while, a specific region of chicken nuclear 5-aminolevulinate synthase gene was used as a target for the PCR amplification in chicken. The PCR conditions, i.e., primer, dNTPs and $MgCl_2$ concentration were optimized and the PCR cycling programme especially the annealing temperature was standardized for

each species. Using species-specific primers (20 pico mol) each, the PCR was performed at a common annealing temperature of 55°C for 30 cycles.

The cattle specific PCR assay yielded a specific product of 381 bp which was common for both *Bos indicus* and *Bos Taurus.* Further, PCR amplification was confirmed in six breeds of cattle (Sahiwal, Holstein Frisian, Jersey, Deoni and Khilari) including a cross-bred. Detection limit of 0.1 pg of template DNA was recorded in the present study which indicated a very high sensitivity of the assay compared to the hitherto reported sensitivity of the techniques using the conventional PCR for the detection of cattle.

Similarly, specific amplification of mitochondrial D loop of the buffalo showed a product of 534 bp. This product was highly specific to the buffalo with a sensitivity of 10 pg. Further, the buffalo specific assay was found suitable even in the cooked (up to autoclaving) and adulterated (up to 0.1%) meat samples.

In sheep assay, using single pair of sheep specific primers multiple PCR products were observed due to the occurrence of heteroplasmy in the sheep mt DNA. Of the four PCR products amplified in raw meat, only two amplicons of size 329 and 404 base pairs were considered diagnostic as these two products were consistent even in the cooked as well as adulterated meat samples.

Caprine mt D loop was amplified using caprine specific primers and the PCR product was evident only in the goat and none other related species including sheep. The assay was highly sensitive with the LOD of 0.1 pg and was capable of detecting even the cooked (up to 121°C) and adulterated (up to 0.1%) tissue samples.

The pig specific primers amplified a portion of mt D loop and gave a single pig specific amplicon of 712 bp in length. This assay was also suitable even in the cooked and adulterated meat samples making it a highly specific assay to porcine DNA.

Mitochondrial 5-amonolevulinate synthase gene was targeted for the identification of chicken species where in a PCR product of 288 bp was observed in chicken only. Further, the amplicon was observed in most of the breed/strains of chicken and not from any other related avian species (turkey, duck, guinea fowl, quail, black kite and parakeet). In the present study, using chicken specific PCR assay a lowest LOD of 10 pg was detected using the conventional PCR.

Cross amplification from the related animal species was checked in all the 6 species-specific PCR assays. For this purpose, as many as 25 species of animals including available breed and strains were checked to exclude the possibility of misidentification of the species. The statistical analysis of the species-specific PCR assays using 2x2 contingency table considering phenotypic species against the PCR detection revealed 100% inclusivity, exclusivity, positive predictivity, negative predictivity and analytical accuracy while the calculated Kappa index was 1 in all the species.

Relative merits of the species-specific PCRs developed in this study include: (i) a single step PCR and gel electrophoresis to conclusively identify a species, (ii) operating protocol and result interpretation was very easy, (iii) these assays could be easily used for the routine analysis of the field samples, (iv) any molecular biology laboratory involved in routine PCR work may take the sample analysis, (v) the assays were optimum even in cooked (up to autoclaving) and adulterated (up to 0.1%) tissues and (vi) high sensitivity (0.1 pg in cattle and goat, 1 pg in sheep and 10 pg in buffalo and pig) of these assays qualify the task of routine sample analysis.

Differentiation of cattle and buffalo species was undertaken by a multiplex PCR using species-specific forward and common reverse primer. The assay can identify cattle and buffalo, where a product of 381 is seen in cattle while a 330 bp product is seen in buffalo, this 51 bp difference in the PCR products could help in their identification and their

differentiation from other species as no product was seen from any other related species tested.

An alternate approach was employed consisting of the isolation of total DNA from the tissues for the identification of those species for which species-specific PCR assays have not been developed. PCR amplification of a segment of mt 12S rRNA gene, its product sequencing and analysis of the sequence through BLAST was used for the identification of a species.

PCR amplification of mt 12S rRNA gene of leopard, Bengal and Siberian tigers, sika deer, goral, muntjak, parakeet, black kite and camel using the universal primers yielded a product of ~456 bp. These amplicons were sequenced and aligned using BLAST (www.ncbi.nlm.nih.gov/BLAST). Based upon the distance, score and alignment these animal species were identified and differentiated from the more closely related species.

In the milieu of social, religious, economic, forensic and public health issues relating to animal species identification, the present work will help the scientists to solve the problem of animal species identification thereby serving the society in a better way.

Appendix

Ammonium acetate (10 mM)

Ammonium acetate	77.08	mg
Distilled water (up to)	100	ml

Assay buffer for Taq polymerase (10X)

$(NH_4)SO_4$	160	mM
Tris-HCl, pH 8.8	670	mM
Tween-20	0.1	%
Magnesium Chloride ($MgCl_2$)	25	mM

Ethidium bromide (10 mg/ml)

Ethidium bromide	10	mg
Distilled water	1	ml

Agarose gel (2%) in TBE

Agarose powder	2	g
TBE (1X)	100	ml
Heat in micro oven for 1 min, cool and then add,		
Ethidium bromide (@ 0.5 µg/ml) 10 mg/ml stock solution	2	µl
Fill to the capacity of gel casting tray with positioned comb.		

DNA extraction buffer (for blood)

Tris buffer (pH 8.0), 1M	5	ml
Sodium chloride, 5 M	40	ml
EDTA buffer, 0.5 M	2	ml
Distilled water up to	500	ml
(Autoclave in batches of 100 ml)		

Tris-HCl 1 M,. pH 8.0

Tris-base	121.1	g
Dissolve in distilled water	800	ml
Adjust the pH to 8 by adding conc. HCl.		
Make the volume to 1000 ml. Autoclave and store		

Lysis buffer (fresh tissue)

Tris-Cl (pH 8.0)	10	mM
EDTA (pH 8.0)	0.1	M
SDS (w/v)	0.5	%
RNase (DNase free, pancreatic)	20	µg/ml

Lysis buffer (cooked tissue)

Tris-Cl (pH 8.0)	10	mM
NaCl	100	mM
SDS (w/v)	1	%
Di-thiothreitol (DTT)	10	mM

Phosphate Buffered Saline (PBS) pH 8.0

Di-sodium hydrogen phosphate	1.15	g
Potassium di-hydrogen phosphate, hydrated	0.20	g
Sodium Chloride	8.0	g
Potassium Chloride	0.2	g
Distilled water to make	1000	ml

RBC lysis buffer

Ammonium chloride	8.3	g
Potassium bicarbonate	1.0	g
0.5 M EDTA (pH 8.0)	299	µl
Distilled water up to 1000 ml (store at 4°C)		

Sodium dodecyl Sulphate (SDS) 10%

SDS	1	g
Distilled water to make	10	ml

RNase (DNase free, 10 mg/ml)

RNase (DNase free)	10	mg
Distilled water	1	ml

Tris-EDTA (TE) buffer 1X, pH 8.0

Tris HCl	10	mM
EDTA	1	mM
Distilled water up to 100 ml and adjust pH 8.0		

Ethylene diamine tetracetate (EDTA), 0.5 M, pH 8.0

EDTA	18.612	g
Distilled water	80	ml
Stir vigorously on a magnetic stirrer to mix.		
Adjust the pH to 8.0 and make the volume to 100 ml.		
Autoclave and store		

TAE buffer (50X)

Tris base	242	g
Glacial Acetic acid	57.1	ml
0.5 M EDTA (pH 8.0)	100	ml
Distilled water (up to)	1,000	ml

TAE buffer (1X)

TAE buffer, 50 X	2	ml
Distilled water up to	100	ml

TBE buffer (10X)

Tris base	108	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40 ml (9.3g)	
Distilled water (up to)	1,000	ml

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Vita

Dr. Nagappa. Karabasanavar, the author of this manuscript was born on 1st March, 1979 in Krishna-Kittur (Belgaum district, Karnataka). He passed his Secondary School Living Certificate (S.S.L.C) examination from S.L.D.K. High School, Krishna-Kittur (Karnataka Secondary Education Examination Board) in 1995 and Pre-University Course (P.U.C) from S.K. Gubbi Science College, Vidyagiri, Dharwad (Department of Pre-University Education, Karnataka) in 1997. He obtained B.V.Sc and A.H. degree in 2002 from Veterinary College Bidar, University of Agricultural Sciences (U.A.S), Krishinagar, Dharwad. Through ICAR-JRF scholarship, he joined Govind Ballabh Pant University of Agriculture and Technology, Pantnagar for M.V.Sc degree with the Major in Veterinary Public Health and Minor in Veterinary Microbiology & Immunology and completed in January-2005. The author worked at Krishi Vigyan Kendra, Bijapur as Training Associate (Animal Science) and later as Assistant Professor at Veterinary College, KVAFSU, Bidar on contractual basis. He then enrolled for Ph. D. programme in July-2005 at G. B. Pant University of Agriculture and Technology, Pantnagar in the Department of Veterinary Public Health with Biochemistry and Animal Biotechnology as minors. He was the recipient of SRF in a DBT funded project and also qualified in ICMR-JRF under research projects scheme (2005). The author also qualified in National Eligibility Test (NET) conducted by CSIR (2005) and ASRB, ICAR, New Delhi (2006). For his PhD work "Young Scientist" award was conferred to him by ISVIB-2008. He has joined as Assistant Professor in the department of Veterinary Public Health at KNP College of Veterinary Science, Shirvsl (MAFSU, Nagpur).

Permanent address:
Dr. Nagappa. Karabasanavar.
At Post: Krishna-Kittur
Taluk: Athani
District: Belgaum
Karnataka- 591 303 (India).
E-mail: *docnagappa@rediffmail.com; nageshsk@yahoo.com; pub.nag@gmail.com*
For details please contact- *docnagappa.weebly.com*

ABSTRACT

Name	**:**	Nagappa Karabasanavar	**Id. No.**	**:**	29840
Semester & Year of admission	**:**	I sem, 2005	**Degree**	**:**	Ph.D
Major	**:**	Veterinary Public Health	**Department**	**:**	Veterinary Public Health
Minor	**:**	1. Biochemistry 2. Animal Biotechnology	**Advisor**	**:**	Dr. S.P. Singh
Thesis title	**:**	"Molecular identification of animal species using Polymerase Chain Reaction based techniques"			

Species-specific PCR assays were developed for the authentic identification of cattle, buffalo, sheep, goat, pig and chicken. The mitochondrial D-loop was targeted in cattle, buffalo, sheep, goat, and pig; while nuclear 5-aminolevulinate synthase gene was used as target for amplification in chicken. The developed species-specific PCR assays yielded products specific to the species studied. In order to exclude the possibility of cross amplification, 25 animal species including different breeds were considered that consisted of mammals, birds, rodent and fish species covering most of the domestic, pet and wild animals. The suitability of the species-specific PCR assays was confirmed in raw (n=20), cooked (60, 80 and 100^{o}C for 30 min), autoclaved (121^{o}C for 30 min) and the micro-oven processed meat and meat products such as kabab, mutton curry, chevon curry, pork sausage, chicken patties, chicken products (samosa, nuggets and loaves). The sensitivity of the PCR was established to be at 0.1% in all species studied for the detection of adulteration and the limit of detection was 0.1 pg in cattle and goat, 1 pg in sheep and 10 pg in buffalo, pig and chicken. Based on the present investigation it was concluded that, the species specific PCR assays developed in this study could be used for the authentication of raw, cooked (up to 121^{o}C) and adulterated (up to 0.1%) animal tissues and their products for the specific identification of cattle, buffalo, sheep, goat, pig and chicken. For the simultaneous detection of cattle and buffalo a multiplex PCR was developed using species-specific forward and common reverse primers. Further, for the identification of an unknown species an alternative approach was used, that involved-isolation of total DNA from the cells, PCR amplification of a region of mitochondrial 12S rRNA gene, sequencing of the PCR amplicon and analysis of the sequence. Using this approach, nine species of animals (leopard, Bengal and Siberian tigers, goral, muntjak, sika deer, camel, parakeet and a black kite) were unambiguously identified. This research work presents novel diagnostic primers for 6 animal species and validates the sequence analysis as a tool for identification animal species.

(S.P. Singh)
Advisor

(Nagappa Karabasanavar)
Author