# 2 LITERATURE REVIEW

## 2.1 Epidemiology of African Trypanosomosis

Human African Trypanosomosis (HAT) has re-emerged as a major health threat to rural poor in several African countries. It is extending from the Southern part of Sudan through Uganda and Congo to Northern Angola (Welburn et al., 2001). According to the 2000 WHO report, there are 55 million people at risk of disease in 36 countries of sub-Saharan Africa. However, only 3 to 4 million of these people are under surveillance (Welburn and Maudlin, 1999; Gibson et al., 2002).

Tsetse flies (Diptera: Glossinidae) are important vectors of African trypanosomes, the causative agents of sleeping sickness disease in humans as well as trypanosomosis in livestock. Sleeping sickness exists in two forms, either the chronic form (West and Central African form) caused by *Trypanosoma brucei gambiense* or the more acute form (East African form) resulting from infection with *Trypanosoma brucei rhodesiense*. In both forms, there are a wide variety of wild and domestic mammalian species that are identified as actual or potential reservoir hosts (Mehlitz et al., 1982; Mihok et al., 1990).

Reports indicate that about 10 million $km^2$ of Sub-Saharan Africa are infested by tsetse extending through 38 countries and with 30% of the total of 147 million cattle in the continent considered to be at risk of infections (Murray and Gray, 1984).

Transmission of trypanosomosis in livestock is either cyclically (*T. congolense, T. vivax, T. brucei*) by tsetse flies or non-cyclically (*T. evansi*) by haematophagus flies (tabanids and

stomoxys). The exception is infection with *T. equiperdum*, which is transmitted by sexual contact.

During cyclical transmission, tsetse flies acquire infection of bloodstream trypomastigotes from the mammalian host blood (Radostits et al., 2000). The trypanosomes enter the midgut where they transform through lengthwise division into epimastigotes in the cardia. Later they penetrate the haemocel via the peritrophic membrane and the midgut epithelium into the salivary glands or proboscis of the fly where they develop into metacyclics, which are infective. During feeding, these infectious stages are inoculated into the skin of the host (Aksoy et al., 2001).

Pathogenesis of trypanosomosis is initiated when infected tsetse inoculate metacyclic trypanosomes into the skin of the host animal. Parasites multiply for a few days and result in a localized swelling (chancre). They consequently invade lymph nodes and then the bloodstream, where they divide rapidly by binary fission. Following infection by trypanosome, the host produces antibodies against the surface-coat antigens (Stich et al., 2002).

African Animal Trypanosomosis (AAT) is mainly caused by *T. congolense, T. vivax* and *T. brucei*. The disease is considered to be most important in cattle but can also cause serious losses in pigs, camels, sheep and goats. Cattle infected with trypanosomes become chronically ill, characterized by rising body temperature which ranges from 39.4 to 40°C, anaemia, anorexia, swelling of superficial lymph nodes, dullness, inactivation, depression, loss of milk and meat production, and death in un-treated cases (Murray and Dexter, 1988).

## 2.2 Biology and ecology of tsetse

### 2.2.1 Classification

Tsetse flies are classified into Genus: Glossina, Family: Glossinidae, Order: Diptera. The 22 different species of Glossina found in Africa today can be classified into three groups: Morsitans (Savannah species), Fusca (Forest species) and Palpalis (Riverine species) (Table 1). Some of these species are divided again into sub-species due to certain minor but constant differences in their anatomy. All members of a particular species will breed successfully with each other and give fertile offsprings. The species differ in the anatomy of the male genitalia.

**Table 1.** Tsetse species and subspecies (FAO, 1982; Leak, 1999)

| **Morsitans-group** | **Palpalis-group** | **Fusca-group** |
|---|---|---|
| *G. morsitans submorsitans* | *G. palpalis palpalis* | *G. fusca fusca* |
| *G. morsitans centralis* | *G. palpalis gambiensis* | *G. nigrofusca nigrofusca* |
| *G. morsitans morsitans* | *G. fuscipes fuscipes* | *G. nigrofusca* |
| *G. austeni* | *G. fuscipes quanzensis* | *G. medicorum* |
| *G. pallidipes* | *G. fuscipes martini* | *G. tabaniformis* |
| *G. swynnertoni* | *G. tachinoides* | *G. brevipalpis* |
| *G. longipalpis* | *G. pallicera pallicera* | *G. longipennis* |
| | *G. pallicera* | *G. frezili* |
| | *G. caliginea* | *G. severini* |
| | | *G. haningtoni* |
| | | *G. fuscipleuris* |
| | | *G. vanhoofi* |

### 2.2.2 Distribution and habitat

The southern limits of the Glossina distribution in Africa lies north of a line drawn from Benguela in Angola to Durban in the Republic of South Africa, while the northern limits are roughly a line from Dakar in Senegal across to Ethiopia and Mogadishu in Somalia on the east coast (Ford, 1962). Elsen et al. (1990) reported the presence of *G. fuscipes* and *G. morsitans submorsitans* in southwestern Saudi Arabia.

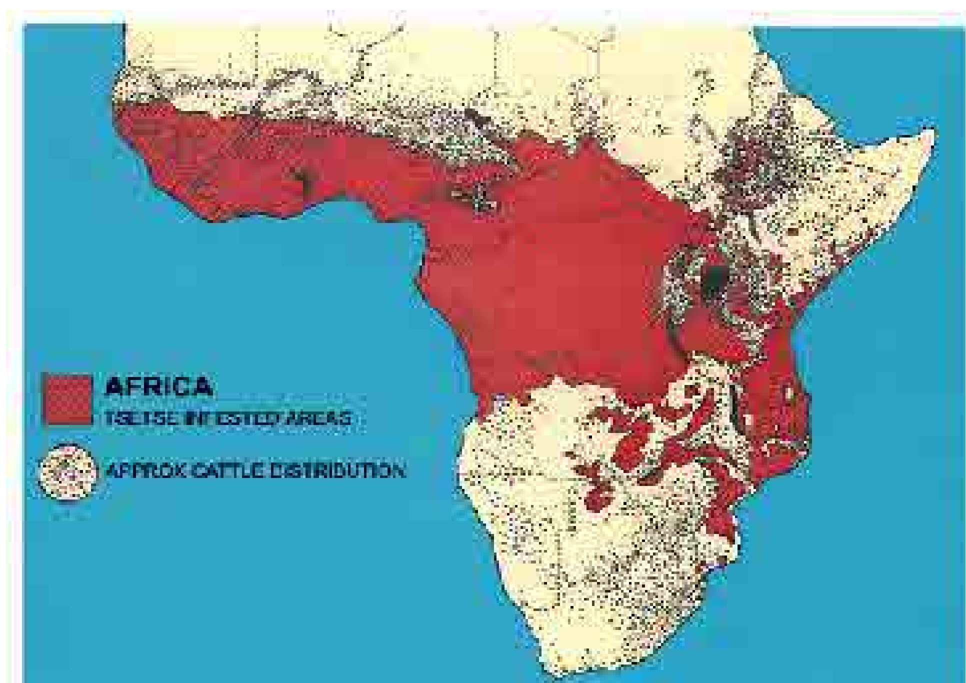


**Figure 1.** Map of distribution of tsetse flies and cattle in Africa (FAO, 1993).

The Morsitans group is not found in the wet areas but is present throughout much of the savannah (grassy woodland) of Africa. Their distribution appears to be limited by cold winter conditions in the north of west and central Africa. The Palpalis group is called the riverine flies, which are associated with local patches of dense vegetation along the banks of rivers and lakes in the arid areas and are also limited to the very humid areas of Africa. On the

other hand, the Fusca group is limited to the more thickly forested areas of Africa (FAO, 1982).

### 2.2.3 Life cycle

The life cycle of tsetse differs from other fly species. Tsetse give birth to a single mature larva unlike other fly species, which reproduce by laying hundreds of eggs. During the mating process, male tsetse flies settle on the back of the female and the claspers at the posterior end of the male abdomen are gripping the end of the female abdomen. Females usually mate only once in their lives but some may mate more than once. Within few hours, the sperms find their way and remain active in the spermathecae for the rest of the life of the female. Older males are better able to mate successfully than very young ones. During mating, a large ball of sperms is deposited in the spermatophore, where the egg is fertilized immediately when the female is 8-11 day old, and then the egg enters the uterus. The larva, which emerges from the egg using a special labral tooth, remains within the uterus. Then the adult fly deposits the larva onto friable soil and after several hours the cuticle becomes sclerotic. The normal development of puparium depends on temperature, which ranges between (22-25°C) and relative humidity (about 80 %) and takes about 4-5 weeks. Tsetse feed every two to three days and the first larval offspring is deposited at about 9-12 days after the female tsetse flies emerge. The life span of females is up to 14 weeks and about 6 weeks for males (FAO, 1982; WHO, 1988).

### 2.2.4 Host preferences

Analysing bloodmeal samples from tsetse by the precipitin test, Weitz (1963) categorized the feeding preferences of the family Glossinidae as follows:

**Group 1.** Glossina feeding mainly on Suidae (*G. swynnertoni, G. austeni, G. tabaniformis, G. fuscipleuris*); **Group 2.** Glossina feeding equally on Suidae and Bovidae (*G. morsitans morsitans, G. morsitans submorsitans*); **Group 3.** Glossina feeding mainly on Bovidae (*G. pallidipes, G. longipalpis, G. fusca*); **Group 4.** Glossina feeding mainly on mammals other than pigs and Bovidae (*G. longipennis, G. brevipalpis*); **Group 5.** Glossina feeding on most available hosts and man (*G. palpalis palpalis, G. fuscipes fuscipes, G. tachinoides*).

By using the complement fixation and ELISA tests, Staak et al. (1986) confirmed the observations of Weitz (1963).

With the use of an ELISA assay, Boreham and Gill (1973) established that 34.4 % of 590 *G. fuscipes* fed on reptiles, while Mohamed-Ahmed and Odulaja (1997) reported that 93.3 % of 520 bloodmeal samples of *G. fuscipes* were from Nile monitor lizards in Kenya. On the other hand, Okoth and Kapaata (1988) observed that the main hosts of *G. fuscipes fuscipes* in Busoga, Uganda, were monitor lizards (*Varanus species*), man (*Homo sapiens*), bush pigs (*Potamochoerus porcus*) and bushbucks (*Tragelaphus scriptus*).

By using the ELISA technique, Clausen et al. (1998) reported that *G. longipennis, G. austeni and G. fuscipes* prefer to feed on bushpigs; *G. morsitans* feeds on warthogs; *G. longipalpis* and *G. fusca* feed mainly on bushbucks; *G. pallidipes* feed mainly on ruminants (buffalo, bushbuck and cattle) and hippopotamus was the main host for *G. brevipalpis* bloodmeals.

Späth (2000) indicated that wild ruminants are the most important source of blood for each Glossina species, but there were significant differences between the nutritional spectra of single fly species; *G. palpalis gambiensis* feeds more often on bushbucks and more significantly on monitor lizards (*Varanus niloticus*). On the other hand, *G. longipalpis*, *G. medicorum* and *G. palpalis gambiensis* feed on wild ruminant species more frequently than *G. palpalis palpalis*. The significant differences in the feeding patterns among the previously investigated species and subspecies revealed microhabitat specialization of the various tsetse groups and different availability of hosts.

Leak (1999) stated that the tsetse flies have the ability to feed on alternative hosts in the absence of the main host, as described in *G. pallidipes*, which can survive on warthogs when bushbucks are scarce.

## 2.3 Bloodmeal identification methods

Serological identification of tsetse bloodmeals depends on the ability of the antisera to recognize only the unique proteins of the host blood (Weitz, 1963; Burkot et al., 1981; Staak et al., 1981, 1986; Clausen et al., 1998; Gomes et al., 2001).

The first serological methods used involved precipitation and inhibition tests, depending on the production of specific antisera with high titres. Serological tests are limited by the specificity of the antisera and by cross-reaction that may occur with antiserum of poor quality, particularly as many hosts of tsetse are closely related species (Emslie and Steinberg, 1973).

### 2.3.1 Precipitin test

One of the earliest serological technique applied in the detection of host specific bloodmeals is the precipitin test. It depends on the interaction between a saline extract of the bloodmeal and a suitable antiserum, usually prepared from rabbits (Weitz, 1960).

A saline extract of the gut contents of the insect is used as antigen, which is layered over a specifically prepared antiserum. The results depend on the interaction between a bloodmeal sample extracted in physiological saline solution and the suitable antiserum used (Weitz, 1963).

### 2.3.2 Haemagglutination inhibition test (HIT)

A high specificity for species and subspecies differentiation was claimed for the haemagglutination inhibition test (HIT) (Weitz, 1960; 1963). The HIT employs the red blood cells, usually obtained from sheep and coated with tannic acid to improve their agglutination capacity. The cells are sensitised with specific serum protein antigen and then agglutinated by antiserum prepared against the protein with which they have been sensitised. Serum from homologous species inhibits the agglutination.

### 2.3.3 Complement fixation test (CFT)

A complement fixation test was adapted by Staak et al. (1981; 1986) to provide species-specific identification of bloodmeals from tsetse flies. This test is based on the principle that it combines complement firmly with the antigen-antibody complex. If a specific antibody is present in the test sample, it will combine with its homologous antigen, and the complement

will become attached to the immune complex. If a non-specific antibody is present in the test sample, it will not combine with its homologous antigen and the unbound complement in the mixture will lead to haemolysis of an indicator system (sheep red blood cells and amboceptor).

### 2.3.4 Enzyme-linked immunosorbent assay (ELISA)

The ELISA has been successfully applied to bloodmeal analysis from tsetse flies, but may present cross-reactivity between phylogenetically closely related species, like domestic pigs, bush pigs and warthogs (Clausen et al., 1998).

This technique was also used for the identification of Anopheles mosquito bloodmeals (Edrissian and Hafizi, 1982; Beier et al., 1988; Gomes et al., 2001) and bloodmeals of Phlebotomine sand flies (Ngumbi et al., 1992).

The efficiency of identification of bloodmeals with ELISA is about 100% up to 20 hours post feeding of the fly (Lombardi and Esposito, 1983). Rurangirwa et al. (1986) reported that the bloodmeals of tsetse could be identified in 100% of fed flies at 40 h post feeding but only in 87.5% at 74 h post feeding.

Gomes et al. (2001) did a comparative study between the precipitin and ELISA tests in bloodmeal detection of Mosquitoes, which fed on feline, canine and human hosts. The authors found better specificity and accuracy in the case of the precipitin test, but ELISA showed a higher sensitivity.

### 2.3.5 DNA based techniques

Recent development in molecular biology have allowed a significant increase in the efficacy and reliability of bloodmeal identification, and DNA-based molecular markers are now being harnessed for typing arthropod bloodmeals. These markers have been used for the analysis of vertebrate hosts DNA obtained from the gut of mosquito (Mukabana et al., 2002). DNA based techniques also have a major significance in the identification of bloodmeals. These techniques include polymerase chain reaction (PCR), restriction fragment length polymorphism analysis (RFLP), DNA probe hybridisation and nucleotide sequencing (Cunningham, 2002).

Recent and accurate methods for arthropods bloodmeal identification include molecular techniques that detect mammalian DNA in the gut of tsetse flies (Boakye et al., 1999; Torr et al., 2001), in ticks (Kirstein and Gray, 1996; Tobolewski et al., 1992) and in mosquitoes (Gokool et al., 1993; Ngo and Kramer, 2003).

Tobolewski et al. (1992) recorded that DNA techniques are a much faster and more reliable way to determine host species than other techniques that depend on antibodies. These techniques are very sensitive and allow for the amplification and identification of the host cytochrome b gene in the gut of ticks. A molecular marker can be used for identification of the zoonotic reservoirs of Lyme Borreliosis and hosts of *Ixodes ricinus* (Kirstein and Gray, 1996) and mosquito bloodmeals (Gokool et al., 1993) by amplification of a specific region of the *cytb* gene. PCR products were amplified and then subjected to digestion with restriction endonucleases. Consequently, this revealed the unique restriction fragment profiles, which allow the taxonomic identification of animals to the genus level.

Identification of bloodmeals in haematophagous Diptera (Glossinidae) by *cytb* sequence is capable of distinguishing between mammalian host samples after several days post-ingestion by tsetse flies (Boakye et al., 1999). In addition, molecular DNA markers have been used for the identification of different tsetse hosts (Torr et al., 2001). According to Lockley and Bardsley (2000), PCR assays allow discrimination of closely related animal breeds.

## 2.4 Molecular techniques, principals and applications for species identification

### 2.4.1 DNA probe hybridization

DNA probe techniques depend on a small sequence of nucleic acid (oligonucleotides) that has been labelled with a radioactive isotope or dye (e.g. enzyme) to locate a complementary nucleotide sequence or gene.

DNA-probes have been developed for the identification of cattle, sheep, goat, horse, deer, pig, chicken and turkey species (Chikuni et al., 1990; Buntjer et al., 1995), and were also used in determining species of raw meat as well as heat processed meat (Baur et al., 1989). The use of DNA probe hybridization provides a reliable and sensitive technique to detect the mixture of pork with beef (Wintero et al., 1990).

Ebbehoj and Thomsen (1991 a, b) demonstrated species differentiation of raw and heat-treated mixtures of pork and beef using labelled total genomic DNA from pork as a probe to determine the amount of pork in the samples. The relative accuracy of the determinations was about 20% for large contents of pork, but in small contents it was about 20-100%. The

same authors reported that the technique had some problems in the detection of mixtures of meats of closely related species.

### 2.4.2 Amplified fragment length polymorphism analysis (AFLP)

Amplified fragment length polymorphism analysis (AFLP) is based on the selective PCR amplification of restriction fragments from a total restriction enzyme digestion of genomic DNA. Oligonucleotide adapters are ligated to the restriction fragments. The selective amplification is achieved using amplimers designed from both the restriction enzyme recognition sequence and the adapter sequence, extending a short way into the same restriction fragments. Only those primers that match the restriction fragment at their 3´ end are amplified if a 3´-exonuclease deficient thermostable DNA polymerase is used (Savelkoul et al., 1999).

The AFLP analysis has the ability to provide individual human locus characterizations from dessicated arthropods (Replogle et al., 1994). AFLP is a useful technique for the estimation of the divergences of different species, such as wild and domestic animals, and yields a direct estimation of genetic diversity among and within domestic breeds (Savelkoul et al., 1999).

### 2.4.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is an enzymatic process by which a specific region of DNA is replicated repeatedly to yield several million copies of a particular sequence (Pääbo, 1989; Saiki et al., 1988). It is an *in vitro* technique, which allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA. It is

achieved by use of oligonucleotide primers, which are short, single-stranded DNA molecules that are complementary to the ends of a defined sequence of a DNA template (Newton and Graham, 1997).

Amplification of a DNA fragment requires a pair of oligonucleotide primers of known sequences, which are complementary to DNA sequences flanking the amplified region. Therefore, it has become possible to identify one species among others if a DNA fragment characteristic for this species is absent in the genomes of other species (Guzow-Krzeminska et al., 2001).

PCR was used to amplify homologous segments of mitochondrial DNA (mtDNA) from more than 100 animal species, including mammals, birds, amphibians, fish and some invertebrates. Universal primers are directed towards the conserved regions of *cytb* gene of various animal species (Kocher et al., 1989).

A single PCR technique has the ability to detect mosquito bloodmeals for up to 3 days after feeding using species-specific primers, and further distinguish between mammalian and avian bloodmeals (Ngo and Kramer, 2003). The amplification of specific regions within the *cytb* gene in vertebrate DNA, using *cytb* primers, could be applied for bloodmeal identification from tsetse flies (*G. palpalis*) (Boakye et al., 1999) and from maggots (fly larva) (Wells et al., 2001). Moreover, Kirstein and Gray (1996) reported that the PCR assay could be used for the identification of bloodmeals from ticks, using these primers, which amplify specific regions of the *cytb* gene in several animal species.

The PCR is also a useful technique for detecting vertebrate species using different biological samples, such as blood, semen, hairs, saliva, dried shed skin and feathers (Hershfield et al., 1994; Parson et al., 2000). It is also used for the identification of human bloodmeals from mosquitoes (Ansell et al., 2000; Chow-Shaffer et al., 2000). Moreover, it can be used to identify food components using sets of primers to amplify species-specific regions of mitochondrial *cytb* gene (Calvo et al., 2001a; Calvo et al., 2002; Herman, 2001; Maudet and Taberlet, 2001; Meyer and Candrian, 1996; Bottero et al., 2002).

Masri et al. (2002) reported that PCR has been used for the identification of salmon fish from another species of fish, using species-specific primers that amplify the growth hormone. This technique could also be used for amplification of the *cytb* gene using one pair of universal primers, producing a 359 bp fragment in all flatfish species (Céspedes et al., 1998). Species-specific amplification of a region of the mitochondrial cytochrome b gene was used to discriminate bluefin tuna (*Thunnus thynnus*) from Atlantic bonito (*Sarda sarda*) (Lockley and Bardsley, 2000) and the differentiation of different species of fresh water fish was reported by Nguyen et al. (2002).

Species identification was carried out by nucleotide sequence analysis of the cytochrome b (*cytb*) gene, using the amplified conserved region of *cytb* gene from 44 different animal species, covering 5 major vertebrate groups (mammals, birds, reptiles, amphibians and fish) (Parson et al., 2000; Wang et al., 2000). This method could distinguish between sheep species and subspecies (Hiendleder et al., 1998).

The PCR primers developed to amplify a segment of the mitochondrial control region from blood DNA could also be used for amplification of mtDNA obtained from the hair of an

Asian elephant (*Elephas maximus*) and African elephant (*Loxodonta africana*) (Goossens et al., 1998; Greenwood and Pääbo, 1999; Vigilant, 1999).

The presence of water-soluble melanin is primarily responsible for the colour seen in skin, hair and eyes, and acts as a PCR inhibitor, due to inhibition of the Taq DNA polymerase (Yoshii et al., 1992; 1993; Hühne et al., 1999; Pfeiffer et al., 1999). The same authors observed that removal of water-soluble melanin contaminating DNA could be done by the adsorption of melanin to Bio-Gel or by adding bovine serum albumin (BSA).

Matsunaga et al. (1999) reported a quick and simple method for species identification by PCR assay, by using species-specific primers (cattle, pig, chicken, sheep, goat and horse). PCR primers are designed as a forward primer on a conserved DNA sequence in the mitochondrial *cytb* gene and reverse primers on species-specific DNA sequences for each species. Species-specific primers have been used to detect fresh water eels (Lin et al., 2002). A region of the *cytb* gene of the ostrich (*Struthio camelus*) was amplified, using a species-specific primer pair that permits identification of the ostrich and differentiate it from the emu (*Dromaius norvaehollandiae*) (Colombo et al., 2000). In addition, Montiel-Sosa et al. (2000) stated that pork meat and fat could be detected in meat products, using specific PCR primers for pig.

### 2.4.4 Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)

Random amplified polymorphic DNA (RAPD), also known as arbitrarily primed PCR, allows the detection of polymorphisms without prior knowledge of the nucleotide sequence. The method utilizes short primers of arbitrary nucleotide sequence that are annealed in the

first few cycles of PCR at low stringency. The low stringency of the early cycles ensures the generation of products by allowing priming with mismatches between primers and template. The subsequent PCR cycles are performed at a higher stringency after generation of some initial products that now have ends complementary to the primers (Newton and Graham, 1997).

The RAPD method was used to generate fingerprint patterns of pork, chicken, duck, turkey and goose meats (Calvo et al., 2001b). It was used for the identification of two different populations of cultured Korean catfish (Yoon and Kim, 2001; Rego et al., 2002) and also for authentication of seafood products (Bossier, 1999). Moreover, the method has been used for the identification of species of the *Anopheles minimus* group in Southeast Asia (Kengne et al., 2001).

### 2.4.5 Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP)

Restriction fragment length polymorphism analysis (RFLP) is a technique in which species may be differentiated by analysis of patterns derived from cleavage of their DNA. If two species differ in the distance between sites of cleavage of particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. Restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequence, depending on the particular enzyme used. Enzyme recognition sites are usually 4 to 6 base pairs in length.

A PCR-RFLP method using universal primers complementary to a conserved region of the vertebrate *cytb* gene was adapted to identify bloodmeal donors of vector arthropods (Kirstein and Gray, 1996).

Tartaglia et al. (1998) reported that the PCR-RFLP analysis is a specific, rapid, simple and highly sensitive technique, which is successfully used to detect and identify bovine-derived meats and bone meals in ruminant feeds. This method detects the presence of the bovine mtDNA in feedstuffs at level less than 0.125%. Moreover, it is possible to identify various animal species by analysis of the *cytb* gene of mtDNA (Murray et al., 1995; Ishida et al., 1996; Wolf et al., 1999 b; Zehner et al., 1998).

Lenstra et al. (2001) recorded that PCR-RFLP is the most suitable technique for species identification of meat products, especially in case of degraded samples and also for the detection of admixture; the technique, however, is sensitive to contamination. Rajapaksha et al. (2002) reported that an amplified conserved region of the mitochondrial *cytb* gene could be used to differentiate between meats from important wild animals in Sri Lanka.

PCR-RFLP analysis provides a simple, rapid and less expensive method, alternative to sequencing of PCR products, and allows the discrimination of species, even when the template DNA may be degraded or contains only traces of DNA (Lenstra et al., 2001; Burger et al., 2002). PCR-RFLP was also used for the identification of 22 animal species by amplification of a 359 bp region located in the *cytb* gene and then digesting the amplified PCR products, using *Hae*III and *Hinf*I. The *cytb* PCR-RFLP is a promising method for the determination of both cooked and uncooked tissues (Partis et al., 2000). It has been used to detect the species from which milk used in cheese production originates (Branciari et al.,

2000). The technique can be applied to meat species identification in marinated and heat-treated or in fermented products and also to differentiate between closely related species of vertebrates (Meyer et al., 1995). In addition, the assay has been used for the identification of porcine, caprine and bovine meats (Sun and Lin, 2003).

Analysis of conserved regions of mtDNA with PCR-RFLP was used for the identification of Hake species in food products (Quinteiro et al., 2001), to distinguish among closely related avian species (Lovette et al., 1999), for the detection of fish species (Mackie et al., 1999; Cocolin et al., 2000; Hold et al., 2001; McDowell and Graves, 2002), for several types of sturgeon caviar (Wolf et al., 1999 a), clam species (Fernández et al., 2000; 2001), Anchovy and Gilt Sardines (Sebastio et al., 2001), Salmon species (Carrera et al., 1999; Russell et al. 2000) and flat fish species (Sotelo et al., 2001).

PCR-RFLP was further used for the identification of ostrich meat, using analysis of mitochondrial *cytb* gene fragments (Abdulmawjood and Bülte, 2002). RFLP was used to detect 12 species of petrels or tube-nosed sea birds representative of four family groups and revealed clear restriction profiles (Millar et al., 2000).

#### 2.4.5.1 Mitochondrial DNA (mtDNA)

The mitochondria are cellular organelles that have the function of oxidative phosphorlation. The mtDNA is a double-stranded, circular and closed molecule of 16.500 nucleotides (Anderson et al., 1981; Castro et al., 1998). The complete (mtDNA) molecule of the pig (*Sus scrofa*) has 16.679 nucleotides (Ursing and Arnason, 1998b), in the hippopotamus 16.407 nucleotides (Ursing and Arnason, 1998a), in the ostrich (*Struthio camelus*) 16.591

nucleotides (Härlid et al., 1997) and in crocodiles about 16.642 nucleotides (Janke and Arnason, 1997).

Mitochondrial genomes of all vertebrate animals analysed to date have the same 37 genes, whose arrangement in the circular DNA molecule varies only in the relative position of few genes that encode 13 mRNAs, 22 tRNAs and 2 rRNAs. Mitochondrial DNA (mtDNA) is maternally inherited and complete gene arrangements have been published for 58 chordate species and 29 non-chordate species (Mindell et al., 1998; Boore, 1999).

The high rate of substitution makes mtDNA particularly valuable in studying the relationships in recently diverged lineages. It is widely used in systematic studies to resolve divergences at many taxonomic levels, e.g. as a molecular marker for interfering phylogenetic relationship at various levels within the fish family Cichildae (Farias et al., 2001; Prychitko and Moore, 2000).

The mtDNA sequencing is a useful technique for forensic identification of human and animal biological specimens (Pierpaoli et al., 1999; Dimo-Simonin et al., 2000; Luiz et al., 2002). The mtDNA sequences have been used to identify dogs by analysis of DNA extracted from hair samples that have been stored for a long time (Schneider et al., 1999; Lebedeva et al., 2000) and different Asian elephant species (Fleischer et al., 2001). The use of mtDNA control region sequences has been employed for human identification based on teeth, skeletal remains and hairs (Higuchi et al., 1988; Lutz et al., 1996; Allen et al., 1998; Pfeiffer et al., 1999; Bender et al., 2000). There were no differences observed between mtDNA sequences obtained from hair shafts and those from blood and saliva samples (Hühne et al., 1999).

#### 2.4.5.2 Cytochrome b (*cytb*) gene

Cytochrome b is a central redox catalytic sub-unit of the quinol, responsible for the transmembrane electron transfer and it is one of the most useful genes for phylogenetic work (Esposti et al., 1993). It is approximately 1140 bp long (Katsumi et al., 1997). A full-length *cytb* pseudogene, found in rodents, has apparently been translocated from mitochondria to the nuclear genome; the pseudogene differs from its mitochondrial counterpart at 201 of 1143 sites (17.6%) (DeWoody et al., 1999).

Ettore et al. (1996) showed that the differences among *cytb* sequences of wild and domestic pigs can be used to obtain a phylogenetic tree of the Suiformes (Artiodactyla).

The mitochondrial encoded *cytb* gene was used as a molecular marker for the discrimination of several animal species, which act as hosts for *Ixodes ricinus* (Kirstein and Gray, 1996). The highly conserved *cytb* gene can be used for the detection of a variety of mammals, such as cattle, sheep, goat, cat, dog, humans and for birds (Ron et al., 1996; Hsieh et al., 2001). According to Branicki et al. (2003) *cytb* sequence analysis can be used as a method of species identification.