Parasitol Res (2005) 97: 247–254
DOI 10.1007/s00436-005-1410-y

ORIGINAL PAPER

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PCR-RFLP analysis: a promising technique for host species identification of blood meals from tsetse flies (Diptera: Glossinidae)

Received: 10 May 2005 / Accepted: 19 May 2005 / Published online: 6 July 2005

Abstract A polymerase chain reaction with the restriction fragment length polymorphism (PCR-RFLP) method using universal primers complementary to the conserved region of the cytochrome b gene (*cyt b*) of the mitochondrion DNA (mtDNA) of vertebrates was applied to the identification of the origin of blood meals in tsetse flies. Blood samples from ten potential tsetse hosts of the family bovidae (cattle, water buffalo, red buffalo, waterbuck, springbok, goat, sheep, sable antelope, oryx and dik-dik) were included in this study. Sites for appropriate restriction endonucleases cuts were chosen by pairwise alignment of the amplified 359 bp fragments. A flow chart of endonucleases digestion using three restriction enzymes (e.g. *Taq*I, *Alu*I and *Hin*dII) for the unequivocal identification of the respective bovid species was developed. A number of additional non-specific DNA fragments attributed to the co-amplification of cytochrome b pseudogenes were observed in some species (e.g. in red buffalo and dik-dik after digestion with *Alu*I) but did not hamper assignment of bovid species. The detection rate of host DNA in tsetse by PCR-RFLP was 100, 80, 60 and 40% at 24, 48, 72 and 96 h after in vitro feeding, respectively. Identification of the last blood meal was possible even when tsetse had previously fed on different hosts.

Keywords *Glossina* · Blood meal identification · Bovidae · Cytochrome b · PCR-RFLP

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Introduction

Detailed knowledge of the feeding behaviour of tsetse flies on their various vertebrate hosts is considered to be a prerequisite for a successful tsetse and trypanosomosis control programme. For this reason serological techniques like the precipitin and haemagglutination test (Weitz 1960, 1963), the complement fixation test (Staak et al. 1981, 1986) and the enzyme-linked immunosorbent assay (ELISA) (Clausen et al. 1998) have been developed to identify the source of vertebrate blood in the intestinal tracts of wild tsetse flies. Up to now, however, some problems remain with the identification of phylogenetically closely related species, which may result in a high percentage of samples being identified only to the family level (e.g. Suidae, Bovidae) but not to the exact species taxon (Clausen et al. 1998).

Through the last decade numerous developments in molecular biology techniques have allowed a considerable increase in the accuracy and the reliability of analytical methods for the identification of vertebrate species. Such methods were primarily developed for the accurate identification of species in samples of meat (Chikuni et al. 1990; Ebbehoj and Thomsen 1991; Meyer et al. 1994; Buntjer et al. 1995), but they are also considered to be useful for the precise characterization of sources of blood meals obtained from haematophagous arthropods (Boakye et al. 1999; Torr et al. 2001; Mukabana et al. 2002; Ngo and Kramer 2003). In this context, it appears that the mitochondrial DNA (mtDNA) is a preferred target in identifying the species origin since mtDNA contains a high proportion of evolutionary-caused nucleotide substitutions making it particularly valuable as a discriminatory molecule in studying the relationships between closely related vertebrates. Moreover, the early identification of a standard set of universal primers directed towards conserved regions of the mitochondrial cytochrome b (*cyt b*) gene from vertebrates, by Kocher et al. (1989), enables an adequate PCR amplification of relevant nucleotide sequences especially from highly

processed foodstuff or largely digested DNA samples found in haematophagous arthropods. In particular, the combination of the polymerase chain reaction with the restriction fragment length polymorphism analysis (PCR-RFLP) is a widely used method for the accurate determination of species origin of samples taken from meat, foodstuff (Céspedes et al. 1998; Wolf et al. 1999; Carrera et al. 1999; Partis et al. 2000; Lenstra et al. 2001; Abdulmawjood and Bülte 2002; Sun and Lin 2003) and dairy products (Branciari et al. 2000; Lanzilao et al. 2003; Stefos et al. 2004). By contrast, studies on species identification of blood meal donors in vector arthropods using the PCR-RFLP fingerprinting method are rather scarce. Kirstein and Gray (1996) devised a PCR-RFLP approach that allows the determination of reservoir hosts of Lyme disease (*Borrelia burgdorferi*) in *Ixodes ricinus* ticks for at least 200 days after larval blood ingestion. By targeting a 638 bp sequence of the *cyt b* gene, they were able to identify the animal taxon down to the genus level.

The aim of the present study was, therefore, to adapt the PCR-RFLP as a complementary technique to ELISA for the identification of blood meals from tsetse flies down to the species level. The specific objectives of the study were (i) to develop an appropriate flow chart using restriction enzymes that unequivocally enables the species differentiation within a given vertebrate family (e.g. family Bovidae) and (ii) to evaluate the detection rate of host DNA in the gut of tsetse after in vitro feeding.

Materials and methods

Animals' origin and collection of blood samples

Reference blood samples from species of the family Bovidae were obtained from various zoos in Germany. The blood samples were collected by jugular vein puncture using EDTA-coated vacutainer tubes (Terumo, Germany). One millilitre was aliquoted into 1.5-ml tubes (Eppendorf, Germany) and kept at −20°C until use.

Preparation of blood meals from tsetse

Glossina palpalis palpalis

Pupae, kindly provided by the International Atomic Energy Agency (IAEA, Vienna, Austria), were maintained in cages in an insectary at 25–28°C and 70–80% relative humidity.

Control group

The intestinal tracts of teneral flies ($n = 5$) were dissected about 24 h after emergence and squeezed onto filter paper using the lateral part of a pair of forceps and left to dry at room temperature (RT). Afterwards, the filter papers were stored at −20°C until use.

Non-teneral groups

Forty-eight hours after hatching, the teneral flies were allowed to feed on sheep blood using a silicone membrane at 37°C (Bauer and Wetzel 1976). After 24-h periods (24, 48, 72, 96 and 120 h) the guts of fed tsetse ($n = 5$ each) were dissected and squeezed onto filter paper as described before. A further group of flies was first fed on sheep blood followed by a second feeding on goat blood 48 h after first feeding. They were dissected 24, 48 and 72 h after ingestion of the goat blood. The isolated guts of flies were prepared as described before.

DNA extraction

EDTA blood

The DNA from vertebrate hosts was extracted according to Higuchi (1989). A 250-µl EDTA blood sample was mixed with 250 µl lysis buffer (0.32 M Sucrose, 0.01 M Tris, 0.005 M $MgCl_2$, 1% Triton X-100, pH 7.5) and centrifuged at 13,000 *g* for 25 s. After removal of the supernatant, the pellet was washed three times by vortexing with 500 µl lysis buffer followed by spinning down at 13,000 *g* for 25 s. The final pellet was resuspended in 250 µl one time PCR buffer (10 mM Tris, 50 mM KCl, 0.1% Triton X-100, pH 8.3) containing 1.5 µl of a stock solution of proteinase K (10 mg/ml) (Boehringer Mannheim, Germany) and incubated at 56°C for 1 h. Finally, the proteinase K was inactivated at 95°C for 10 min. The DNA concentration was calculated spectrometrically by means of a Gene Quant DNA Calculator (Amersham Pharmacia Biotech, Germany). The extracted DNA samples were stored at −20°C until use.

Blood meal samples from tsetse

The punched filter paper section containing the blood smear from the gut of tsetse was eluted in one time PCR buffer, left at RT for 1 h, during that time the mixture was vortexed every 15 min. Subsequently, 250 µl of the solution were mixed with 250 µl lysis buffer, washed with lysis buffer and treated with proteinase K as described before. Finally, the DNA samples were stored at −20°C until use.

DNA amplification (PCR)

The primers used for PCR are complementary to the conserved region of the *cyt b* gene of vertebrate's mtDNA. They are derived from the primers L14841 and H15149 described by Kocher et al. (1989):

Forward (*cyt b1*): 5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′

Reverse (*cyt b2*): 5′-GCC CCT CAG AAT GAT ATT TGT CCT CA-3′

Amplifications were carried out in 25 µl PCR reaction buffer (10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 100 µM each of the four dNTP's, one unit of AmpliTaq Gold polymerase (Applied Biosystems, Germany) containing 0.5 µM each of the primers and 5 ng DNA template. At each PCR a negative control containing distilled water instead of DNA was run in parallel. The reaction buffer was overlaid with one drop of mineral oil and centrifuged at 10,000 *g* for 15 s. The PCR reaction was carried out in a Trio-Thermoblock (Whatman Biometra, Germany) adjusted to the following parameters: DNA denaturation and polymerase activation at 95°C/10 min, 35 cycles at 94°C/30 s, annealing at 52°C/30 s, extension at 72°C/45 s, final extension at 72°C/5 min 10 µl of each PCR product was visualized by submarine minigel electrophoresis through 2% agarose gel in one time electrophoresis buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 8.0) stained with EtBr and run at 60 V for 1 h.

Sequence analysis and identification of cleaving sites

To obtain sequence information on the *cyt b* gene of the different bovid species tested, the retrieval tool of the National Centre of Biotechnology Information (NCBI) was used (Entry: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = Nucleotide). Alignment data of the expected *cyt b* region (359 bp) with the consensus strain (*Bos taurus*) are given in Fig. 1.

Sites for restriction enzymes that cleave the 359 bp fragment were identified by means of the programme NEB Cutter Version 2.0 (Vincze et al. 2003). Cleaving sites for some candidate enzymes located by the search programme are shown in Table 1. The final selection criteria for species identification were based on the minimum number of enzymes required to produce discriminating restriction fragment profiles. Conditions were met by the enzymes *Taq*I, *Alu*I and *Hin*dII, recognizing the target sequences T↓CGA, AG↓CT and GTY↓RAC (Y = T or C; R = A or G). To obtain data on species origin from flies, which fed subsequently on sheep and goat blood, the amplified *cyt b* fragment was subjected to cleavage using restriction endonuclease *Nde*II (target sequence: ↓GATC) because this enzyme has been judged to be discriminatory for sheep (31/115/213 bp) versus goat DNA (115/244) (Meyer et al. 1995).

Endonuclease digestion and restriction fragment length polymorphism (RFLP) analysis

Prior to digestion, the PCR products were purified using the commercially available QIAquick PCR purification kit (Qiagen, Germany). Endonuclease digestion was carried out in 20 µl reaction volumes, comprising 8 µl of the purified PCR products, two units of each restriction enzyme and 2 µl ten times reaction buffer as enclosed by the supplier. Incubation

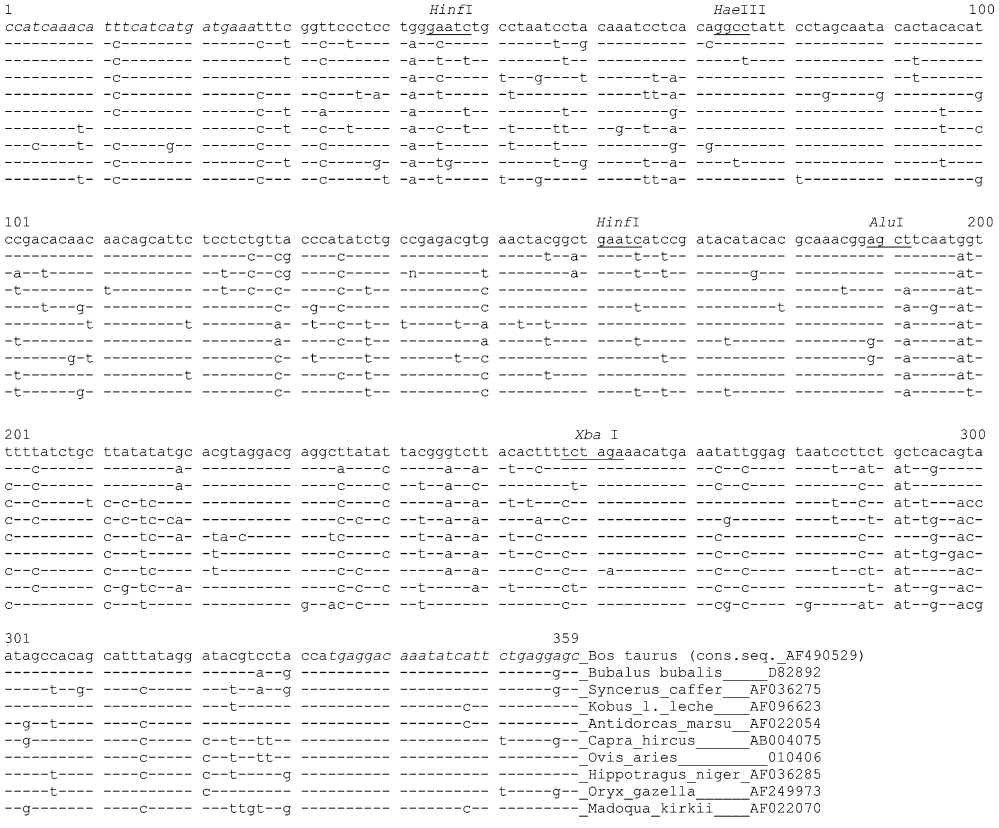

Fig. 1 Nucleotide sequence of a 359-bp *Cyt b* fragment of a *B. taurus* cattle (consensus sequence) aligned with sequence data from nine different species of the family Bovidae. The corresponding accession numbers are given according to the NCBI database. The PCR primer sequences are shown in *italics*. Position of different restriction endonucleases that cleave cattle DNA are *underlined*

Table 1 Restriction map of common restriction endonucleases for a 359-bp mitochondrion *cyt b* fragment of ten species of the family Bovidae

	Restriction enzymes (cleaving sites)						
	*Taq*I T↓CGA	*Alu*I AG↓CT	*Hin*dII GTY[a] ↓R[b] AC	*Xba*I T↓CTAGA	*Hin*fI G↓AN[c] TC	*Hae*III GG↓CC	*Nde*II ↓GATC
Cattle (*B. taurus*)	359	169/190	359	101/258	44/117/198	74/285	359
African water buffalo (*B. bubalis*)	168/191	169/190	359	101/258	359	74/285	115/244
Red buffalo (*S. caffer nanus*)	168/191	55/114/190	150/209	359	359	126/233	115/244
Waterbuck (*Kobus l. leche*)	359	359	150/209	359	161/198	74/126/159	115/244
Springbok (*A. marsupialis*)	166/193	359	150/209	359	359	74/126/159	115/244
Goat (*C. hircus*)	141/218	359	359	101/258	161/198	55/74/230	31/115/213
Sheep (*O. aries*)	359	359	359	359	63/296	74/126/159	115/244
Sable antelope (*H. niger*)	168/191	55/304	150/209	359	161/198	74/126/159	115/244
Oryx (*O. gazella*)	359	55/304	150/209	359	161/198	126/233	359
Dik-dik (*M. kirkii*)	168/191	100/259	150/209	359	126/233	55/74/230	78/281

Sequence data of bovid species were obtained from the NCBI database and generated by the programme NEB Cutter Version 2.0 (Vincze et al. 2003; http://tools.neb.com/NEBcutter2/index.php)
[a] Y = T or C
[b] R = A or G
[c] N = A, T, C or G

conditions were chosen according to the manufacturers' recommendations (Roche Diagnostics, Bio Lab, Boehringer Mannheim, all Germany). To visualize the restriction patterns, aliquots of 8 µl digested samples mixed with 2 µl loading buffer (four times TBE buffer, Ficoll-400 10% (w/v), SDS, 0.4% (w/v), bromophenol blue 8 mg) were subjected to submarine minigel electrophoresis through 4% Nusieve agarose gel (Biozyme, Germany) in one time TBE buffer (1 mM Tris, 1 M boric acid, 20 mM EDTA, 50 mM $MgCl_2$ $6H_20$) stained with EtBr and run at 60 V for 1 h.

Results

PCR-RFLP analysis of DNA from species of the family Bovidae

The amplification of a fragment encoding a 359-bp sequence of the *cyt b* gene yielded the expected amplification product in all tested bovid species.

To obtain characteristic restriction profiles, the amplified *cyt b* fragments were subjected to digestion by

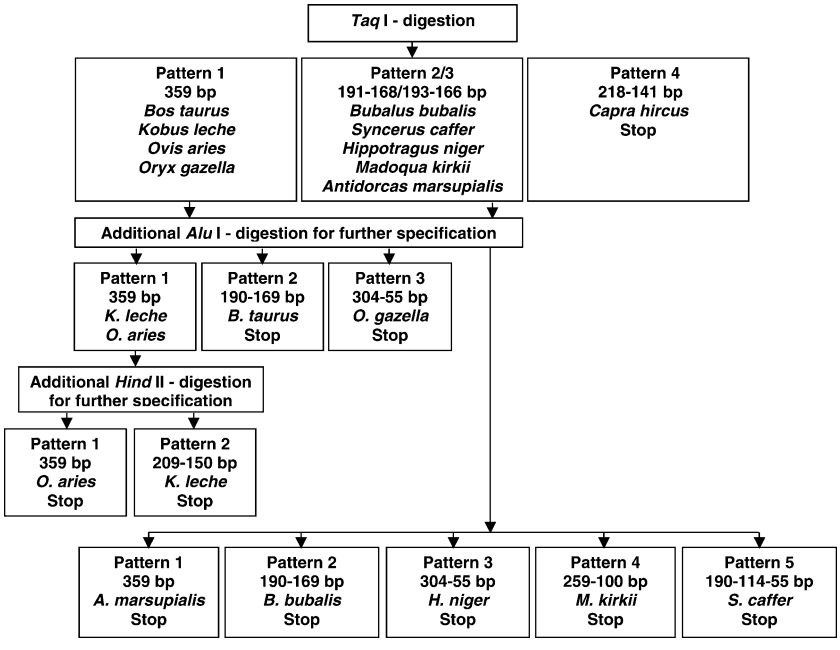

Fig. 2 Proposed flow chart of restriction endonuclease digestions for the unequivocal identification of ten animal species of the family Bovidae

restriction endonucleases. The selection of appropriate restriction endonucleases is facilitated by the DNA sequence data of vertebrates *cyt b* genes already published (NCBI sequence data base), which enables, therefore, both a detailed sequence alignment and the preparation of species—specific restriction maps using the screening programme NEB Cutter Version 2.0. Based on these data, an optimized flow chart has been established that allows direct identification of the bovid species by a combination of usually two (*Taq*I and *Alu*I) but not more than three (plus *Hin*dII) restriction enzymes (Fig. 2).

Practically, the analysis of *Taq*I restriction pattern showed the expected fragments of 218 and 141 bp for goat (*Capra hircus*) and the calculated digestion fragments of 191 and 168 bp for water buffalo (*Bubalus bubalis*), red buffalo (*Syncerus caffer nanus*), sable antelope (*Hippotragus niger*) and dik-dik (*Madoqua kirkii*) as shown in Fig. 3. The *Taq*I fragments of springbok (*Antidorcas marsupialis*) are close in length (193–166) and, therefore, failed to accurately discriminate the springbok from the preceding species (e.g. goat, buffalo). According to the sequence analysis, no cleavage of PCR products from cattle (*B. taurus*), sheep (*Ovies aries*), waterbuck (*Kobus leche*) and Oryx (*Oryx gazella*) was achieved.

The additional *Alu*I digestion allows the subsequent differentiation of eight out of ten bovid species (Fig. 3). As anticipated from the flow chart, the *Alu*I digestion led to fragments of 169 and 190 bp in cattle and water buffalo, while those of oryx and sable antelope were 55 and 304 bp in length (Fig. 3, lane 8, 9). No *Alu*I restriction sites were found for waterbuck, springbok, goat and sheep (Fig. 3, lane 4–7).The *Alu*I digestion of the red buffalo (Fig. 3, lane 3) resulted in the expected restriction pattern (55/114/190 bp) and a non-specific fragment of 285 bp. Digestion of the *cyt b* amplicon of the dik-dik yielded the expected fragments (100/259 bp) but also several non-expected fragments (55/190/304 bp).

The *Hin*dII digestion allows differentiation between waterbuck and sheep DNA (Fig. 3, lane 4 and 7) because in both species discriminatory restriction sites for *Taq*I and *Alu*I do not exist. As anticipated, digestion of the DNA amplicon of the waterbuck with *Hin*dII resulted in fragments of 150 and 209 bp (Fig. 3, lane 4), whereas no *Hin*dII restriction sites were found in the *cyt b* PCR products from sheep DNA (Fig. 3, lane 7). All other bovid species showed either no (cattle, water buffalo and goat) or one uniform restriction site (red buffalo, sable antelope oryx and dik-dik) resulting in fragments of 150 and 209 bp.

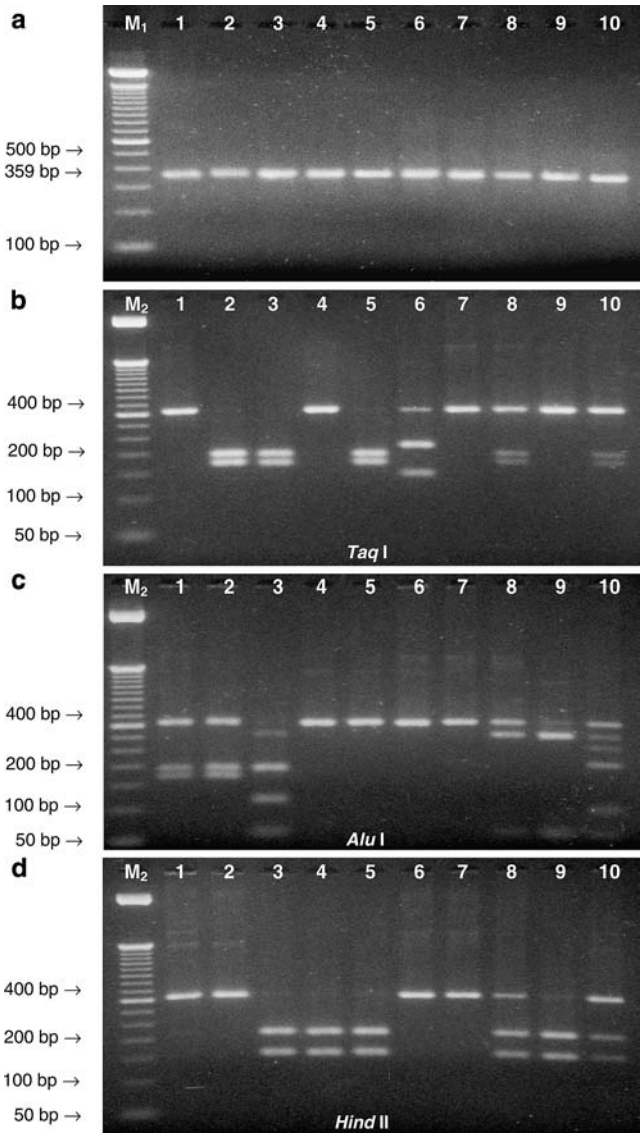

Fig. 3 Restriction profiles of an amplified 359 bp fragment of *Cyt b* obtained from different species of the family Bovidae **(a)** after digestion with *Taq*I **(b)** *Alu*I **(c)** and *Hin*dII **(d)**. *Lane 1* Cattle (*B. taurus*), *Lane 2* Water buffalo (*B. bubalis*), *Lane 3* Red buffalo (*S. caffer nanus*), *Lane 4* Waterbuck (*K. leche*), *Lane 5* Springbok (*A. marsupialis*), *Lane 6* Goat (*C. hircus*), *Lane 7* Sheep (*Ovis aries*), *Lane 8* Sable antelope (*H. niger*), *Lane 9* Oryx (*O. gazella*), *Lane 10* Dik-dik (*M. kirkii*). M_1 100 bp marker, M_2 50 bp marker

PCR amplification of DNA from unfed/fed tsetse

When DNA prepared from the gut of teneral tsetse (*Glossina palpalis palpalis*) was subjected to amplification, no signals were visible indicating that the dipteran DNA does not interfere with the specific amplification of the vertebrates *cyt b* DNA (Fig. 4: lane 1–5). On the other hand, DNA prepared from guts of tsetse flies fed on sheep blood and used as template in the amplification reaction led to the vertebrate specific *cyt b* product of 359 bp. The PCR products of the expected length were detected in all tsetse flies ($n = 5$) killed 24 h after feeding (Fig. 4: lane 7–11), while 4 out of 5 PCR reactions gave the expected signals 48 h after feeding (Fig. 4: lane 12–16). Specific amplifications were also achieved in four out of five samples, when tsetse were killed 72 h post-feeding; a very weak signal was noted only in one tsetse (Fig. 4: lane 18). Positive signals were detectable in three out of five fed flies at 96 h, although one signal was too

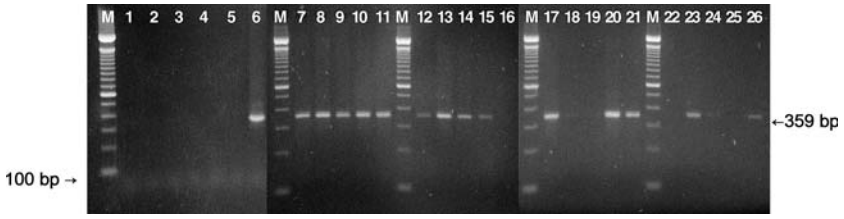

Fig. 4 *Cyt b*-specific PCR (359 bp product) conducted on tsetse flies before and after feeding on sheep blood. The time course post-feeding runs from the *left* to the *right*: *Lanes 1– 5* teneral tsetse flies, *Lane 6* positive control DNA (sheep DNA). *Lanes 7– 11* PCR reaction from tsetse up to 24 h post-feeding, *Lanes 12– 16* PCR reaction from tsetse uop to 48 h post-feeding, *Lanes 17– 21* PCR reaction from tsetse up to 72 h post-feeding, *Lanes 22– 26* PCR reaction from tsetse up to 96 h post-feeding. *M* 100 bp marker

weak for restriction enzyme analysis (Fig. 4: lane 24). Very weak or no blood meal signals could be found 120 h after feeding impeding processing of the products by restriction enzymes (data not shown).

In order to determine which host can be identified when tsetse are fed subsequently on two animals at an interval of 48 h, the amplified product originating from flies fed on sheep and goat blood were subjected to cleavage using the restriction enzyme *Nde*II (Fig. 5). Results show that the digestion of the amplification products yielded the characteristic restriction profile from the goat (31, 115, 213 fragments) irrespective of the sampling time (i.e. 24, 48 or 72 h) after the second feeding.

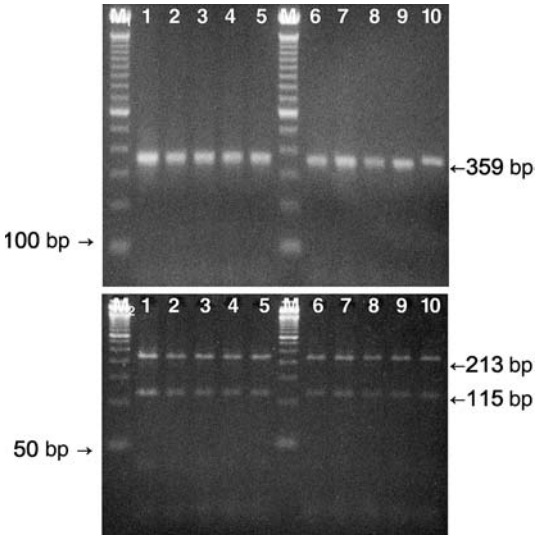

Fig. 5 *Cyt b*-specific amplification products (359 bp) generated from tsetse, which have subsequently fed on sheep and goat blood 48 h apart. *Top*: *Lanes 1– 5* Blood meals from tsetse 24 h after last feeding on goat blood. *Lanes 6–10* Blood meal from tsetse 48 h after last feeding on goat blood. M_1 100 bp ladder. *Bottom*: The same amplicons after digestion with *Nde*II. The resulting restriction profiles reveal the calculated pattern of goat DNA (213/115/31 bp) The expected 31 bp restriction fragment is not easily visible due to its small size. M_2 50 bp ladder

Discussion

By using a molecular genetic approach, the present study aimed to develop an optional follow-up method for the precise identification of tsetse blood meals from bovidae from the family level down to the species taxon. For the differentiation between species we chose the combined PCR-RFLP, which was based on the amplification of a short variable region of the mitochondrial *cyt b* gene using universal primers complementary to a mainly conserved region of the gene (Kocher et al. 1989). This was followed by selective restriction enzyme cleavage within the variable region. This technique has already been established as a valuable DNA fingerprint method for the differentiation of material from closely related animal species especially in food products (Meyer et al. 1995; Céspedes et al. 1998; Partis et al. 2000; Russell et al. 2000; Bellagamba et al. 2001). The advantage of this technique is the availability of nucleotide sequence entries of the *cyt b* gene from a constantly growing number of vertebrates in public data bases (Parson et al. 2000), allowing both a rapid sequence alignment of species and the identification of cutting sites for potential restriction enzyme without prior costly sequencing of amplified *cyt b* products from the animal species in question.

Due to the fact that the family Bovidae was judged to be the family with the largest number of different species known as potential hosts for tsetse feeding, we decided to include DNA specimen from ten animal species of the family Bovidae in this study. As expected, *cyt b* sequence entries from all the selected bovid species could be found in the database of the NCBI (http://www.ncbi.nlm.nih.gov/) so that prior to experimental PCR-RFLP analysis an adequate sequence alignment of the fragments to be amplified was feasible.

By using the *B. taurus* 359 bp *cyt b* fragment as a consensus sequence, the alignment and comparison of the primer region from the ten bovid species that are selected shows only minor differences between the animals revealing not more than four mismatches within the forward primer (*H. niger*) and only two mismatches within the reversed primer (*C. hircus*, *Oryx gazella*). Consequently, all prepared and isolated DNA samples showed a clear signal of 359 bp after amplification. Up to now, we have examined more then 30 DNA samples isolated from skin, hair or peripheral blood of unrelated vertebrates (e. g. hippopotamus, rhinoceros, African elephant, dromedary camel, giraffe, lizard, domestic pig, lion, donkey, crocodiles, rabbits, chimpanzee and

snakes), without observing any amplification failures using the universal *cyt b1*/*cyt b2* primers (data not shown).

Sites for many restriction enzymes that cut the 359 bp sequence were identified by means of the free available programme NEB Cutter Version 2.0 offered from New England Biolab Incorporation (http://tools.neb.com/NEBcutter2/index.php). This tool can list most of the commercially available restrictions enzymes that cleave a given sequence. Based on these data a theoretical flow chart of PCR-RFLP has been proposed allowing differentiation between bovid species. Three different restriction enzymes (*Taq, Alu*I and *Hin*dII) were found to be appropriate for species identification, but it should be noted that many bovids can be classified after application of only two endonucleases. These findings are similar to those of Kirstein and Gray (1996) who reported that the RFLP analysis of a 638 bp PCR *cyt b* fragment with only two restriction enzymes (*Hae*III and *Dde*I) allowed the taxonomic identification of the potential zoonotic reservoir of Lyme Borreliose down to the genus level.

Experimentally, the PCR-RFLP analysis using the selected restriction enzymes largely yielded the predicted results and the bovid species could accurately be identified according to the flow chart (Fig. 2). *Taq*I reveals the characteristic RFLP patterns (359, 191–168/193–166 and 218–141 bp) and discrimination of all but two bovids was subsequently possible by means of the *Alu*I digest (359, 304–55, 259–100, 190–169 and 190–114–55 bp). Both endonucleases, however, failed to distinguish between waterbuck (*K. leche*) and goat (*C. hircus*). In our approach we suggest therefore, the additional digestion with *Hin*dII, since only the waterbuck reveals a single cleaving site in the *cyt b* target fragment (209–150 bp).

Unexpected fingerprints with a surplus of additional bands after digestion were noticed in a few animal species (*Alu*1: red buffalo, dik-dik). Neither the use of higher amounts of restriction enzymes (up to ten units) nor the extension of the incubation period improved the cleaving results. Such unexpected DNA fingerprints after PCR-RFLP of a *cyt b* gene fragment, however, were also reported by several other authors applying the PCR-RFLP analysis and are commonly attributed to the co-amplification of *cyt b* pseudogenes (Meyer et al. 1995; Partis et al. 2000; Lenstra et al. 2001). Pseudogenes reflect non-functional nuclear copies of mtDNA segments (syn. termed "numts") and may appear in many sizes, from all types of mtDNA sequences and bear a varying degree of similarity to their mitochondrial counterparts (Zhang and Hewitt 1996; Bensasson et al. 2001). It is thought that they are evolutionarily incorporated into nuclear genome during the repair of chromosomal breaks by non-homologous recombination (Bensasson et al. 2001). The inadvertent co-amplifications of numts via PCR are suspected to affect in particular studies on phylogeography and taxon relationships where mtDNA is one of the main sources of information (Pereira and Baker 2004). However, it should be stressed that identification of the species is not really hampered by numts when the predicted restriction fragment are also visible.

The universal *cyt b* primers were also used to assess the detection limit of vertebrate DNA imposed by progressing digestion of the imbibed blood meal in the gut of tsetse. As previously reported (Torr et al. 2001), the universal *cyt b* primer applied in our study completely failed to amplify mtDNA from unfed tsetse so that the RFLP-analysis of a blood meal will not be impeded by amplified DNA of tsetse origin. In our study it was possible to identify donor-specific PCR products from fed tsetse up to 96 h post-ingestion. Moreover, adequate amplifications of the *cyt b* gene from blood meal donors were 100% reliable up to 24 h post-feeding but still attainable from 60–80% of tsetse 48–72 h post-ingestion. The period during which we were able to satisfactorily amplify the *cyt b* fragment of the blood meal DNA is therefore slightly less than earlier findings using a serological technique. Using ELISA, blood meal donors were 100% identifiable up to 40 h post-feeding in teneral tsetse and 87.5% identifiable up to 74 h post-feeding. It is noteworthy, however, that non-tenerals digested the species-distinguishing components faster than tenerals and only 67.5 and 50% of the blood meals could be identified up to 40 and 74 h post-feeding, respectively (Rurangirwa et al. 1986). Whether in our experiments the use of non-teneral tsetse would negatively influence the outcome of amplification is presently not known but warrants further investigation.

To evaluate, whether PCR-RFLP enables the precise identification of subsequent blood meals in tsetse, some flies were allowed to feed initially on sheep followed by a blood meal on goats 2 days later. After digestion with the restriction enzyme *Nde*II, only the characteristic restriction patterns of goat DNA were recorded, indicating that this technique could be used to detect the last host of tsetse.

In conclusion, our results indicate that the PCR-RFLP analysis is a promising method for host species identification in tsetse flies. It should be stressed that present costs of PCR-RFLP limits the large-scale use of this method and ELISA will continue to be the primary standard for blood meal identification up to the family level. However, in case where detailed information on host species of tsetse is required, complementary PCR-RFLP analysis has proven to be a useful technique.

Declaration

The authors declare that all experiments performed in the study do comply with the current laws of Germany.

Acknowledgements We wish to thank Prof. Dr. Klaus Eulenberger (Leipziger Zoo), Dr. Wolfram Rietschel (Zoologisch-Botanischer Garten Wilhelma Stuttgart), PD. Dr. Kai Fröhlich (Institute for Zoo and Wildlife Research Berlin) and Dr. Andreas Ochs (Zoo-

logischer Garten Berlin) for providing blood samples from different bovid species used in the study. Special thanks to Mrs. Angelika Wiemann for excellent technical assistance. This work was funded by the Ständige Kommission für Forschung und wissenschaftlichen Nachwuchs (FNK) of the FU Berlin.

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