3 MATERIALS AND METHODS

3.1 Study animals

Blood samples were collected from 33 wild and domestic vertebrate species, consisting of 25 genuses, 19 families and 11 orders from different sites of Germany (Table 2). These animals were classified systematically according to Stuart and Stuart (1997).

3.2 Collection of test samples

3.2.1 Collection of blood samples

Whole blood samples were collected from the vertebrates by jugular venipuncture into 10 ml EDTA-coated vacutainer tubes (Terumo GmbH, Eschborn, Germany). Two millilitres EDTA-blood was aliquoted into 2x 1.5 ml Eppendorf tubes. The samples were kept frozen in a freezer at – 20°C until tested using PCR.

3.2.2 Collection of hair and skin samples

Hair and skin samples were collected from animals that are difficult to bleed such as crocodile, lion, elephant and tortoise. The samples were taken under aseptic conditions in clean, sterile Eppendorf tubes and using gloves to avoid the contamination of samples with another DNA and stored at –20°C until use.

3.3 Extraction of DNA from vertebrate hosts

3.3.1 DNA extraction from EDTA blood

DNA from vertebrate hosts was obtained following the extraction protocol described by Higuchi (1989). Two hundred and fifty microlitres of EDTA blood was mixed with 250 µl lysis buffer (0.32 M Sucrose, 0.01 M Tris, 5 mM $MgCl_2$, 1% Triton X-100, pH 7.5). The mixture was centrifuged at 13.000 g for 25 sec. The supernatant was removed and the pellet washed with 500 µl lysis buffer. The centrifugation and washing were repeated twice. The final pellet was resuspended in 250 µl 1 x PCR buffer (10 mM Tris-Hcl (pH 8.3), 50 mM KCl, 0.1 % Triton X-100). 1.5 µl of proteinase-K (10 mg/ml) was added and then mixed by vortexing. The samples were incubated at 56ºC for 1 hour and at 95ºC for 10 min. (to inactivate the proteinase-K) and stored at -20ºC until use. The DNA concentration was estimated by means of a spectrophotometer (Gene Quant Calculator®, Amersham Pharmacia Biotech, Freiburg, Germany).

3.3.2 DNA extraction from hair and skin of vertebrate hosts

DNA from hair shafts and skin samples of host species was extracted according to the method described by Sullivan et al. (2002). Briefly, approximately 2 cm of hair shaft including the root or skin was placed into sterile microcentrifuge tubes. Several washings with sterile distilled water were conducted. After that the samples were put into a sterile microcentrifuge tube containing 250 µl extract mixture (20 % Chelex 100® Resin, 250 mg proteinase-K and 40 mM Dithiothreitol). The samples were then incubated at 37ºC overnight. The mixture was boiled for 8 min to inactivate proteinase-K. The samples were centrifuged at 13.000 g for 3 min to pellet any undigested hair, skin and the Chelex 100® Resin. The supernatant, which contained DNA, was transferred into a clean sterile tube and subjected to purification with Qiagen Tissue Kit® (Qiagen GmbH, Hilden, Germany). The mixture was transferred into a DNeasy® spin column placed in a 2 ml collection tube and centrifuged at

6.000 g for 1 min. The DNeasy® spin column was placed in a new 2 ml collection tube. Then, 500 µl buffer AW1 was added and centrifuged for 1 min at 6.000 g and the DNeasy® spin column placed in a new 2 ml collection tube. In addition, 500 µl buffer AW2 was added and centrifuged for 3 min at 13.000 g until the DNeasy® membrane dried. The DNeasy® spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl buffer AE was pipetted directly onto the DNeasy® membrane. Consequently, the tube containing buffer and DNA samples was incubated at room temperature for 1 min. The mixture was centrifuged for 1 min at 6.000 g. The supernatant containing the extracted and purified DNA was stored at –20ºC until use.

3.4 Preparation of bloodmeals from tsetse

3.4.1 Maintenance of tsetse *in vitro*

Tsetse pupae (*Glossina palpalis palpalis*) were obtained from the International Atomic Energy Agency (IAEA) laboratory in Vienna, Austria. They were placed in clean and sterile cages to avoid contamination of each bloodmeal with other sources of DNA. Pupae were kept at about 24.5 ± 0.5ºC and 85% relative humidity (Deloach and Taher, 1983). After hatching, the adult flies were divided into two experimental groups.

Experimental group 1 (unfed group)

The intestinal tracts of unfed tsetse flies were removed about 24 h after hatching, squeezed onto filter paper (Whatman No. I) and left to dry at room temperature. The filter papers were stored at –20ºC until tested.

Experimental group 2 (fed group)

The flies were fed on blood of known origin (sheep blood) through a silicone membrane forty-eight hours after hatching as described by Bauer and Wetzel (1976) and Bauer and Aigner (1978). Prior to feeding the flies, defibrinated and sterile sheep blood is poured on a sterile plastic membrane, warmed by an electric unit to keep the membrane surface at 37ºC. The blood pool is then covered with a silicone membrane 40 x 40 cm, 0.5 mm thick (Silicone Release agent AK, Wacker Chemie GmbH, Munich, Germany) on which the cages containing the flies are placed. At various times post-feeding (24, 48, 72, 96 and 120 h), flies were dissected (Figure 1). The intestinal tracts were removed and smeared onto Whatman filter paper, dried and stored at –20ºC until processed. The second group of flies were initially fed on sheep blood and later maintained on goat blood 48 h after the first feeding. These flies were later dissected 24, 48 and 72 hours after feeding on goat blood.

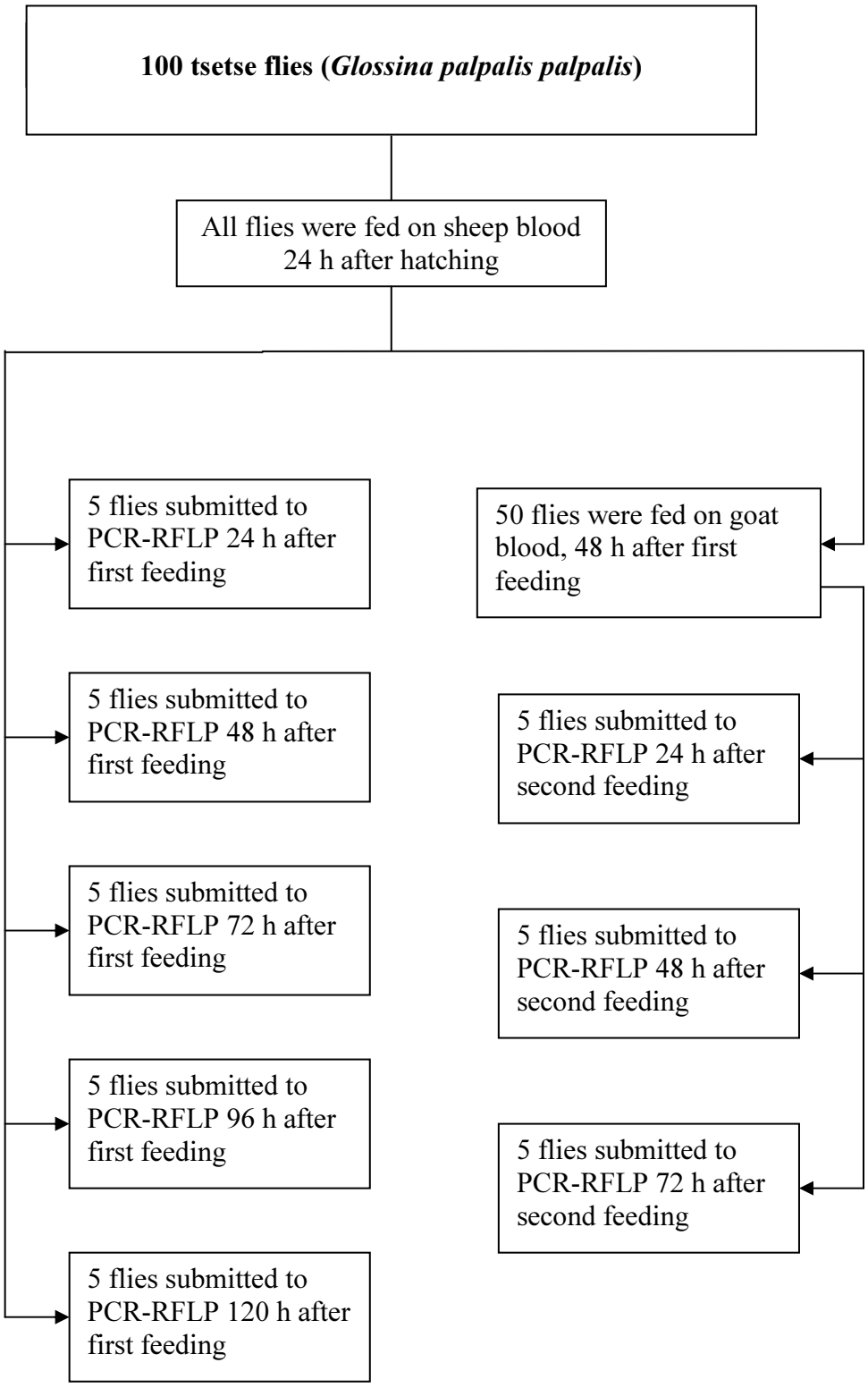

Figure 2. Bloodmeal collection protocol from fed tsetse flies

3.4.2 Preparation of bloodmeal smears

The flies were immobilised by placing them in a refrigerator (4ºC) for 10 min. The abdomen was cut off with a scalpel under a light microscope. The guts of the flies were removed from the abdomen and spread onto filter paper (Whatman No. 1), using the side of a forceps blade. The dissecting instruments were washed and sterilized with 0.2% sulphuric acid, rinsed in distilled water (3x) and wiped with a clean paper towel to avoid contamination. The blood smears were left at room temperature until they dried. The filter papers containing the blood samples were immersed in absolute antiseptic solution such as acetone, chloroform and ether for one hour to sterilize bloodmeals and prevent spreading of diseases, e.g. when transferring blood smears from one country to the other for testing (Boreham, 1976). The samples were stored at –20ºC until use.

3.4.3 DNA extraction from blood smears

The section of filter paper containing the blood smear was cut out and eluted in 500 µl 1 x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 % Triton X-100), left for one hour at room temperatures and mixed every 15 minutes. Two hundred and fifty microlitres of the supernatant were 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). The mixture was centrifuged at 13.000 g for 25 sec. The supernatant was poured out and the pellet washed with 500 µl lysis buffer. Centrifugation and washing was repeated two times. The final pellet was resuspended in 250 µl 1 x PCR buffer. Then, 1.5 µl of proteinase-K (10 mg/ml) was added and mixed by vortexing. The samples were incubated at 56ºC for 1 hour and at 95ºC for 10 min (to inactivate the proteinase-K) and stored at -20ºC until use. The DNA concentration was estimated by means of a

spectrophotomer, Gene Quant Calculator® (Amersham Pharmacia Biotech, Freiburg, Germany) and stored at –20ºC until use for PCR amplification.

3.5 DNA amplification

Amplifications of DNA for *cytb* PCR were carried out in 25 µl reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of the four dNTP´ s (dATP, dCTP, dGTP, dTTP), 1 unit of Ampli Taq Gold® polymerase (Applied Biosystems, GmbH, Darmstadt, Germany), approximately 5 ng DNA and 0.5 µM of each of the oligonucleotide primers. For the amplification of DNA prepared from hair or skin, 0.001% of bovine serum albumin (BSA) was added to the PCR reaction mix to overcome the inhibitory effect of melanin pigment (Pfeiffer et al., 1999).

The universal primers used were shown to be complementary to the conserved regions of the mitochondrial cytochrome b gene in vertebrates. The primer sequences used were as follows (Kocher et al., 1989):

Cytochrome b 1 (*cytb* 1): 5´-CCATCCAACATCTCAGCATGATGAAA-3´ Forward primer

Cytochrome b 2 (*cytb* 2): 5´-GCCCCTCAGAATGATATTTGTCCTCA-3´ Reverse primer

The reaction mixture was overlaid with one drop of mineral oil and centrifuged at 10.000 g for 15 seconds. The tubes were transferred immediately into the thermal cycler.

Cycling parameters

PCR was performed by incubating the samples at three temperatures corresponding to three steps (denaturation, annealing and extention) in a cycle of amplification.

The polymerase chain reaction was carried out in a Thermocycler Trio-Thermoblock™ (Whatman Biometra, Göttingen, Germany), programmed to perform a denaturation step at

95ºC for 10 min (to activate the *Taq* Gold® DNA Polymerase), followed by 35 cycles consisting of 30 sec at 94ºC, 30 sec at 52ºC and 45 sec at 72ºC. The last extension step was 5 min longer. The samples were stored at 4ºC until use in the next step.

3.6 Gel electrophoresis

Two percent agarose gel containing 2.5 µl ethidium bromide in 1 x electrophoresis buffer was prepared. The electrophoresis chamber was filled with buffer solution (1 x electrophoresis buffer) until the top surface of the gel submerged by approximately 1 mm (about 400 ml). Two microlitres of the sample buffer was mixed with 10 µl PCR product and centrifuged at 10.000 g for 20 sec. The DNA ladder (100 bp) was prepared. The samples (10 µl) and DNA ladder (10 µl) were placed into the agarose gel wells. The chamber was connected to a 60 Volt power supply and the run was initiated. The separated DNA products were detected using UV transillumination. The PCR products containing a DNA sequence of 359 bp were amplified and later photographed using a Polaroid® camera.

3.7 DNA sequences

The mitochondrial cytochrome b (*cytb*) gene sequences (359 bp fragments) of the respective vertebrates were obtained from the available home page of the National Centre of Biotechnology Information (NCBI):

(http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html)

The fragment sequences obtained from the gene bank data base are listed in Table 3.

3.8 RFLP analysis

For restriction fragment length polymorphism (RFLP) analysis of the *cytb* products, restriction endonucleases were selected from restriction maps of *cytb* sequences of the animal species of the family Bovidae. The selection criteria were based on the minimum number of enzymes required to produce diagnostic restriction fragment profiles. To satisfy these criteria, three enzymes, *Taq* I, *Alu* I and *Hind* II, which recognize the sequences T↓CGA, AG↓CT and GTY↓RAC respectively, were selected (Table 4). Sites for restriction enzymes that cleave a 359 bp-fragment were identified by means of the programme *NEB cutterVI.0* designed by New England Biolab Incorporation (NEB Inc.): http://tools.neb.com/NEBcutter/ .

Before restriction site analysis of the *cytb* gene, the PCR products were purified by placing the products onto one end of Parafilm and drawn with the tip of a pipette to another end to remove the traces of mineral oil present in the product. The second method of purification was through a QIAquick® PCR purification kit (Qiagen GmbH, Hilden, Germany). Five hundred microlitres of buffer PB were mixed with 100 µl PCR sample. The mixture was placed in the QIAquick® column and centrifuged at 6.000 g for 30 second. The sample was washed with 750 µl buffer PE and centrifuged at 6.000 g for 30 seconds. To remove the residues of the buffer PE, the same tube was centrifuged at 6.000 g for an additional 1 min. To elute the DNA sample, 50 µl buffer EB or H_20 was added onto the QIAquick® membrane, the column was centrifuged at 6.000 g for 1 min and the DNA stored at –20ºC.

Endonuclease digestions were performed in 20 µl reaction volumes, which were carried out with 8 µl purified PCR products, 2 µl 10 x enzyme reaction buffer and 2 units of enzyme.

The reaction mixture (volumes given for one sample):

Component	**Volume**
Purified PCR product	8.0 µl
Enzyme reaction buffer (10x)	2.0 µl
DNase and RNase free water	9.8 µl
Restriction enzyme (10 units/)	0.2 µl
Total	20.0 µl

The mixture was incubated at 37ºC (65ºC in *Taq* I) for 4 h or overnight. The digested samples were examined by electrophoresis through 4% NuSieve agarose ultra pure DNA grade agarose (Biozyme, Hessisch Oldendorf, Germany) and I x TBE buffer (Tris-borate/EDTA electrophoresis buffer). In order to recognize species-specific banding patterns, the yielded restriction profiles were compared with a 50 bp DNA molecular size marker. Later, the restriction profiles were photographed using a Polaroid® camera.

Table 2. Genomic DNA bank of domestic and wild species established at the Institute for Parasitology and International Animal Health, FU-Berlin, Germany.

Latin name	English name	German name	Origin
Bovidae (Family)			
Bos (Genus)			
Bos taurus	Cattle	Rind	1
Bos indicus	Cattle	Rind	1
Bubalus (Genus)			
Bubalus bubalis	Water buffalo	Wasser-Büffel	4
Syncerus (Genus)			
S. caffer nanus	Red buffalo	Roter-Büffel	2
Kobus (Genus)			
Kobus leche	Waterbuck	Wasserbock	2
Antidorcas (Genus)			
A. marsupialis	Springbok	Springbock	2
Capra (Genus)			
Capra hircus	Goat	Ziege	1
Ovis (Genus)			
Ovis aries	Sheep	Schaf	1
Hippotragus (Genus)			
H. niger	Sable antilope	Säble-antilope	3
Oryx (Genus)			
O. gazella	Oryx	Oryx	2
Madoqua (Genus)			
M. kirkii	Dik-dik	Dik-Dik	3
Camelidae (Family)			
Camelus (Genus)			
C. dromedarius	Dromedary camel	Dromedar	2
Hippopotamidae (Family)			
Hippopotamus (Genus)			

H. amphibies	Hippopotames	Flusspferd	2
Suidae (Family)			
Sus (Genus)			
S. domesticus	Domestic pig	Schwein	1
Giraffidae (Family)			
Giraffa (Genus)			
G. cameloparadalis	Giraffe	Giraffe	2
Canidae (Family)			
Canis (Genus)			
C. familiaris	Domestic dog	Hund	1
Felidae (Family)			
Felis (Genus)			
F. catus	Domestic cat	Katze	1
Panthera (Genus)			
P. leo	Lion	Löwe	2
Equidae (Family)			
Equus (Genus)			
E. caballus	Horse	Pferd	1
E. asinus	Donkey	Esel	3
E. burchellii	Common zebra	Zebra	3
E. grevyi	Grevy zebra	Zebra	3
Rhinocerotidae (Family)			
Diceros (Genus)			
D. bicornis	Rhinoceros	Nashorn	2
Elephantidae (Family)			
Elephas (Genus)			
E. maximus	Indian elephant	Indischer Elephant	3
Loxodonta (Genus)			
L. africana	African elephant	Afrikanischer Elephant	3
Galiformes (Order)			
Phasianidae (Family)			
Gallus (Genus)			

G. gallus	Chicken	Huhn	1
Leporidae (Family)			
Oryctolagus (Genus)			
O. cuniculus	European rabbits	Kaninchen	1
Varanidae (Family)			
V. Species	Lizard	Eidechsen	3
Colubridae (Family)			
Elaphe (Genus)			
E. guttata	Snake	Schlangen	3
Alligatoridae (Family)			
Caiman (Genus)			
C. crocodiles	Crocodile	Krokodil	3
Hominidae (Family)			
Homo (Genus)			
H. sapiens	Human	Mensch	
Pongidae (family)			
Pan (Genus)			
P. troglodytes	Chimpanzee	Schimpanse	2
Cercopithecidae (Family)			
Papio (Genus)			
Papio hamadryas	Baboon	Pavian	2

1 = Free University of Berlin (FUB).

2 = Zoological Gardens of Berlin, Stuttgart, Leipzig and Munich.

3 = Institute for Zoo- and Wild Animal Research (IZW), Berlin.

4 = Federal Institute for Risk Assessment (BFR), Berlin.

Table 3. The mitochondrial cytochrome b (*cytb*) gene sequences for each investigated animal species are available under the corresponding accession number from the gene bank database http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html

Investigated species	**Accession number**	**Reference sequence**
Bos taurus (Cattle)	AF490528	11907 - 12266
Bos indicus (Cattle)	AF419237	69 - 428
Bubalus bubalis (Water buffalo)	D82892	69 - 428
Syncerus caffer nanus (Red Buffalo)	AF036275	69 - 428
Kobus leche (Waterbuck)	AF096623	69 - 428
Ovis aries (Sheep)	AF010406	14227 - 14586
Capra hircus (Goat)	AB004075	69 - 428
Antidorcas marsupialis (Springbok)	AF 022054	69 - 428
Hippotragus niger (Sable antelope)	AF036285	69 - 428
Oryx gazella (Oryx)	AF249973	69 - 428
Madoqua kirkii (Dik-dik)	AF022070	69 - 428
Camelus dromedaries (Arabian camel)	U06426	69 – 428
Hippopotamus amphibius (Hippopotamus)	U07565	69 - 428
Sus scrofa domesticus (Domestic pig)	AB015079	69 – 428
Giraffe cameloparadalis (Giraffe)	X56287	69 - 428
Canis familaris (Domestic dog)	NC_002008	14251 - 14610
Felis catus (Domestic cat)	U20753	15106-15465
Panthera leo (Lion)	AF053052	69-428
Equus caballus (Horse)	D82932	69-428
Equus asinus (Donkey)	NC_001788	69 - 428

Equus grevyi (Grevyi zebra)	X56282	69 - 428
Diceros bicornis (African rhinoceros)	X56283	69 - 428
Elephas maximus (Indian elephant)	AB002412	69 - 428
Loxodonta africana (African elephant)	X56285	69 - 428
Gallus gallus (Domestic fowl)	NC_001323	14964 -15323
Oryctolagus cuniculis (Rabbits)	AF157467	Till 359
Elaphe guttata emoryi (Corn snake)	AF337173	51 - 410
Caiman crocodilus (Crocodile)	NC_002744	14532 - 14891
Homo sapiens (Human)	X93334	14239 - 14598
Pan troglodytes (Common chimpanzee)	NC_001643	14233 – 14592
Papio hamadryas (Baboon)	Y16590	69 – 428
Gopherus polyphemus (Tortoise)	AF020893	72- Till end of sequence

Table 4. Recognition sequences and reaction conditions of restriction enzymes.

No.	Restriction enzymes	Cleavage site	Optimal digestion temperature (°C)	Inactivation temperature (°C)	Isoschizomers and comments
1	***Alu*I**	AG↓CT	37	70	
2	*Cfo*I	GCG↓C	37	65	*Hha*I, *Hin*P1I
3	*Dra*I	TTT↓AAA	37	65	
4	*Dra*III	CACNNN↓GTG	37	65	Star activity
5	*Eco*RI	G↓AATTC	37	65	Star activity
6	***Hae*III**	GG↓CC	37	90	*Bsh*I, *Bsp*KI, *Pa*lI
7	***Hind*II**	GTY↓RAC	37	65	*Hinc*II
8	*Hind*III	A↓AGCTT	37	90	Star activity
9	***Hinf*I**	G↓ANTC	37	80	Star activity
10	***Nde*II**	↓GATC	37	65	*Mbo*I, *Sau*3AI
11	*Pst*I	CTGCA↓G	37	70	Star activity
12	*Rsa*I	GT↓AC	37	65	*Afa*I, *Csp*6I
13	*Sal*I	G↓TCGAC	37	80	Star activity
14	*Ssp*I	AAT↓ATT	37	65	
15	***Taq*I**	T↓CGA	65	90	*Tth*HB8I
16	***Xba*I**	T↓CTAGA	37	70	

Restriction enzymes used in this study in bold.

N = A, T, C or G Y = T or C R = A or G