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Identification of bloodmeals in haematophagous Diptera by cytochrome B heteroduplex analysis

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Abstract. We developed a DNA assay for bloodmeal identification in haematophagous insects. Specific host cytochrome B gene sequences were amplified by PCR and classified on the basis of their mobility in a heteroduplex assay. In the blackfly *Simulium damnosum* s.l. (Diptera: Simuliidae), human cytochrome B DNA sequences were identifiable up to 3 days following ingestion of the bloodmeal. In the tsetse *Glossina palpalis* (Diptera: Glossinidae) collected from tsetse traps in Ivory Coast, bloodmeals were identified as taken from domestic pigs on the basis of their heteroduplex pattern and DNA sequence. Evidently the cytochrome B sequence shows sufficient interspecific variation to distinguish between mammalian host samples, while exhibiting minimal intraspecific variation. The stability of DNA in bloodmeals, for several days post-ingestion by haematophagous insects, allows PCR-HDA assays to be used reliably for host identification.

Key words. *Glossina*, *Simulium*; cytochrome B, heteroduplex analysis, polymerase chain reaction, species identification, Ivory Coast.

Introduction

Identification of the bloodmeal taken by a haematophagous insect provides information on host preferences under natural conditions. The anthropophilic index (percentage feeding on humans) is a vital component of vectorial capacity, while knowledge of other hosts reveals the relative importance of reservoirs of vector-borne zoonotic or enzootic infections.

Contemporary procedures for bloodmeal identification are generally based upon the detection of host antigens by the complement fixation test (Staaq *et al.*, 1981) or by enzyme-linked immunoabsorbant assays (ELISA), using polyclonal antibodies raised against blood components from potential host vertebrates (Chow *et al.*, 1993). This method, however, requires the preparation of immune sera against the blood of each potential host species, a difficult and laborious process. Pre-adsorption steps are also needed to eliminate cross reactions when using this technique (Hunter & Bayly, 1991).

Analysis of DNA sequences of the host species (Gokool *et al.*, 1993) can provide a more specific approach than the serological methods. For example, Kirstein & Gray (1996)

developed a method to identify bloodmeals in the tick *Ixodes ricinus* (L.) (Acari: Ixodidae) based upon polymerase chain reaction (PCR) amplification of a 638-bp fragment of the cytochrome B (cytB) gene encoded in the host vertebrate mitochondrion. Based upon a combination of restriction fragment length polymorphism (RFLP) and hybridization analysis, they used cytB PCR products to classify the tick bloodmeal to the genus level. RFLP and hybridization analyses, however, are cumbersome when analysing large numbers of samples.

Heteroduplex analysis (HDA) is a simple and powerful method of detecting small differences between closely related DNA sequences (Tang & Unnasch, 1995). The PCR amplified DNA to be classified (the sample) is mixed with a closely related probe sequence (the heteroduplex driver), the DNA products denatured and allowed to re-anneal. This results in the formation of four products, consisting of: two homoduplexes resulting from the re-annealing of the completely complementary DNA strands to one another; and two heteroduplex molecules formed from annealing a DNA strand derived from the heteroduplex driver with the complementary strand derived from the sample. Heteroduplex molecules can be separated from homoduplex molecules by electrophoresis on a partially denaturing polyacrylamide gel (Tang & Unnasch, 1997). Mobility of the heteroduplex products is always retarded

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relative to that of the homoduplexes in this system. Moreover, each heteroduplex molecule exhibits a unique mobility, which is dependent on the number, type and position of the mismatches present in the heteroduplex molecule (Zimmerman *et al.*, 1995). HDA has been applied in many situations requiring a method to rapidly classify large numbers of samples. These include distinguishing between, for example, closely related species of blackflies (Tang *et al.*, 1995; Zimmerman *et al.*, 1995), HIV-1 subtypes (Delwart *et al.*, 1995) and different histocompatibility complex alleles in humans (Zimmerman *et al.*, 1993).

We found it possible to amplify host-specific cytB sequences present in the bloodmeals of medically important Dipteran insects. Here we present a method to determine the host bloodmeal origin, based on the mobility of cytB heteroduplex products in an HDA. The utility of this method is demonstrated by the identification of bloodmeals from Afrotropical blackflies of the *Simulium* (*Ewardsellum*) *damnosum* Theobald complex (Diptera: Simuliidae) and wild-caught tsetse, *Glossina palpalis* Robineau-Desvoidy (Diptera: Glossinidae).

Materials and Methods

Specimens

Tissue and blood samples for use in the production of HDA standards were obtained from Abidjan Zoo, Ivory Coast, and from commercial meat vendors and animal facilities of the University of Alabama at Birmingham. The vertebrate species tested included domestic cattle (*Bos taurus* L.), domestic pig (*Sus scrofa* L.), human (*Homo sapiens* L.), domestic chicken (*Gallus gallus* L.), water buffalo (*Bubalus bubalis* L.), antelope (*Gazella spekei* Blythe), cotton rat (*Rattus fuscipes* Waterhouse) and mouse (*Mus musculus* L.). The mosquito *Culex pipiens* L. (Diptera: Culicidae) from our North American strain was used as the negative standard of comparison for insect cytB.

Glossina palpalis tsetse were obtained from tsetse traps set near the village of Simfara (6°37'N, 5°54'E) in the Ivory Coast. *Simulium damnosum* s.l. larvae were obtained from the Nzi river, near Fétékro (6°38'N, 4°42'E), Ivory Coast, and reared to adulthood by the system of Raybould *et al.* (1982). Young adult nulliparous female blackflies were collected from the rearing system, allowed to take a bloodmeal from a human volunteer and kept separately at ambient temperature (25–38°C) for up to 5 days.

DNA extraction

DNA extraction from whole blood-fed flies and from vertebrate samples followed the procedure of Steiner *et al.* (1995). Blood and tissue samples were disrupted by mechanical homogenization in a buffer containing 10 mM Tris-HCl (pH 8.0) 312.5 mM EDTA, 1% (w/v) sodium lauryl sarcosine and 1% polyvinylpyrrolidone. Approximately 15 µl of buffer

was used per 1 mg of tissue. The homogenates were heated to 90°C for 20 min and chilled on ice for 5 min. Samples were subjected to centrifugation at 13 000g for 5 min at room temperature. The supernatant was removed and diluted 20-fold in 10 mM Tris-HCl (pH 8.0) 1 mM EDTA (TE).

PCR amplifications were undertaken in 25 µl of a solution containing 10 mM Tris-HCl (pH 8.3 at 25°C) 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin 200 µM dATP, dCTP, dGTP and TTP, 0.5 µM each primer, 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ, U.S.A.) and 2.5 µl of the DNA template solution, prepared as described above. The sequence of primers used in the PCR was as follows: 5' CCCCTCAGAATGATATTTGTCCTCA 3' and 5' CCATCCAACATCTCAGCATGATGAAA 3'. Reactions began by incubation at 95°C for 3.5 min, followed by 36 cycles comprising 30 s at 95°C, 50 s at 60°C and 40 s at 72°C. The reaction was completed by incubation at 72°C for 5 min.

Heteroduplex analysis (HDA) of PCR products used equal volumes (4 µl) of the sample and driver (water buffalo) PCR products mixed with 8 µl TE and overlaid with 10 µl of mineral oil. The mixture was denatured for 2 min at 98°C and heteroduplex products were allowed to form by slow cooling to room temperature over a period of 30 min. An aliquot (14 µl) of each heteroduplex solution was mixed with 6 µl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). A total of 10 µl of this mixture was loaded onto a 5% polyacrylamide/urea gel (19:1 acrylamide:bis acrylamide, 2.7 M urea) prepared in 108 mM Tris-boric acid (pH 8.3), 2.4 mM EDTA. Electrophoresis was performed on 20 cm × 20 cm Protean II Xii system (Bio-Rad, Hercules, CA, U.S.A.) at 12 mA per gel for 18 h in 90 mM Tris-boric acid (pH 8.3), 2 mM EDTA. Gels were stained in 2 µg/ml ethidium bromide and the homoduplex and heteroduplex patterns were visualized under UV light.

Results

In order to develop a PCR-based assay to identify bloodmeals in haematophagous Diptera, it was first necessary to identify PCR conditions capable of amplifying DNA from the bloodmeal in the presence of relatively large amounts of insect DNA. Furthermore, it was necessary to identify a sequence sufficiently divergent to distinguish between different host species, but conserved enough for unambiguous identification of each potential host species. Previous studies had revealed that the cytB gene might meet these criteria (Kocher *et al.*, 1989; Bartlett & Davidson, 1992). Thus we sought PCR conditions for amplification of vertebrate cytB sequences, but not cytB of insects. As described in the Materials and Methods section, we established PCR conditions to amplify cytB sequences from both mammals and birds, without amplifying the corresponding gene from insect genera representing three important families of haematophagous Diptera, i.e. *Culex*, *Glossina* or *Simulium* (Fig. 1).

When DNA was prepared from extracts of human blood-fed *Simulium damnosum* s.l. and used as a template in the PCR amplification reaction, human DNA was identified in the

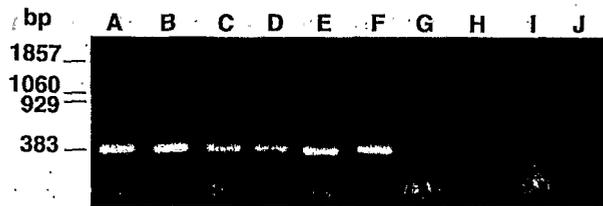


Fig. 1. PCR amplifications of vertebrate and insect *cytB* sequences. Template DNAs for each lane were as follows: A=domestic pig (*Sus scrofa*); B=water buffalo (*Bubalus bubalis*); C=domestic cow (*Bos taurus*); D=human (*Homo sapiens*); E=chicken (*Gallus gallus*); F=chimpanzee (*Pan troglodytes*); G=*Simulium damnosum* s.l.; H=*Culex pipiens*; I=*Glossina palpalis*; J=PCR negative control (no template).

specimens processed up to 72 h postingestion of the bloodmeal (Fig. 2), but no signal was detected in flies kept for longer periods under local conditions of 35°C and 80% r.h.

When potential drivers were tested against *cytB* PCR products derived from different vertebrate species, the *cytB* PCR product from water buffalo was more versatile than those derived from chicken, chimpanzee, human and pig *cytB* PCR products. When tested against itself, as expected, the water buffalo PCR product produced only a homoduplex band (lane 4, Fig. 3). All vertebrate species tested produced HDA patterns readily distinguishable from each other. The PCR product derived from domestic cattle gave a heteroduplex product with the fastest mobility (lane 3, Fig. 3) while those from chicken gave products with the slowest mobility relative to the homoduplex (lane 1, Fig. 3). This is consistent with the expectation that electrophoretic retardation of any heteroduplex product is proportional to the genetic distance between the driver and sample species (Delwart *et al.*, 1994; Tang & Unnasch, 1997).

HDA is an extremely sensitive method for detecting minor sequence differences (Tang & Unnasch, 1995) and it is well known that intraspecific sequence variation exists in the mitochondrial genome of many organisms, including humans (Cann *et al.*, 1984). Such intraspecific variation, if sufficiently large, might interfere with the interpretation of results obtained in the HDA assay described above. To determine if this was the case, DNA from two or more individuals of three species of potential host animals was examined for intraspecific variation in the HDA. No intraspecific variation (data not shown) was seen in the *cytB* HDA from cattle ($n=2$, from Africa and North America), water buffalo ($n=2$) and pigs ($n=7$).

In contrast, some intraspecific variation was noted for human samples tested in the *cytB* HDA. Of 368 individuals from five ethnic groups (Africans, African Americans, Asians, European and North American Caucasians) tested in the *cytB* HDA, 96% (354) gave *cytB* products that were indistinguishable in the HDA. In the remaining 14 individuals, minor variations in mobility of the *cytB* products were detectable (Fig. 4). This variation was insufficient to interfere with the correct identification of the sample as human derived (Fig. 4).

To confirm the utility of the HDA for identification of bloodmeals, DNA was extracted from two female *S. damnosum*

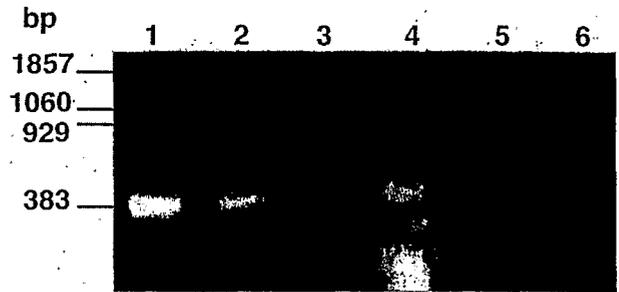


Fig. 2. Time course of detection of bloodmeals in blackflies (*Simulium damnosum* s.l.) fed upon a volunteer and held at ambient temperature for varying periods of time. DNA was extracted from the flies, and *cytB* sequences amplified from the bloodmeal as described in Materials and Methods. Lane 1=positive control; Lane 2=fly held for 24 h following ingestion of the bloodmeal; Lane 3=fly held for 48 h; Lane 4=fly held for 72 h; Lane 5=DNA extracted from an unfed fly; Lane 6=PCR negative control.

s.l. that had blood fed on a human volunteer, and five *G. palpalis* tsetse flies collected in the village of Sinfara, Ivory Coast. In the *cytB* PCR, both of the blackflies and three of the tsetse produced detectable PCR products that were then classified using the HDA assay. As expected, both blackfly specimens gave heteroduplex patterns corresponding to human DNA, whereas the three tsetse bloodmeals gave PCR products identical in mobility to those obtained from domestic pig DNA (Fig. 5). To confirm the identity of these tsetse hosts, the remaining PCR products from the three tsetse bloodmeals were subjected to DNA sequence analysis. The DNA sequence obtained from the bloodmeal PCR products was found to match that of the pig *cytB* gene sequence deposited in the Genbank sequence database (Accession number X56295), confirming the HDA identification.

Discussion

The results presented above demonstrate that it is possible to use vertebrate-specific PCR primers to amplify DNA present in a bloodmeal from haematophagous Diptera. Furthermore, the identity of the bloodmeal can be established by subsequent analysis of the PCR product using HDA. The PCR-HDA assay is somewhat more complicated to apply than is the case for ELISA-based assays (e.g. Chow *et al.* 1993) to identify bloodmeals in field-collected insects. PCR HDA, however, has some advantages over ELISA. First, in order to unambiguously identify a given bloodmeal with ELISA it is necessary to produce polyclonal antisera against the blood of the host in question. Second, it is also often necessary to carry out pre-absorption steps against the blood antigens of other species before the specificity of the antibody reagent can be ensured. Third, it is necessary to test the specimen with antisera produced against all potential hosts in order to unambiguously identify the bloodmeal. In some cases, this is simply impossible, given that the supply of antigen obtained from a given insect specimen may be limited. Finally, it may not be



Fig. 3. HDA analysis of *cytB* PCR products from different species. HDA was carried out using a PCR product derived from water buffalo as a heteroduplex driver. Lane 1=chicken; Lane 2=domestic pig; Lane 3=domestic cow; Lane 4=water buffalo; Lane 5=gazelle; Lane 6=human; Lane 7=cotton rat; Lane 8=mouse.

possible to identify a particular bloodmeal if a suitable antibody is not available.

In contrast to the ELISA-based method, the HDA-PCR-based method relies upon the mobility of the heteroduplex molecule formed by hybridizing sample and driver PCR products to give the identification. Thus, with an optimized heteroduplex driver and a collection of reference DNA samples, it is possible to identify the bloodmeal using a single assay. Furthermore, bloodmeals for which standards are not available will be detected in the HDA, because they will produce heteroduplex products with novel mobilities. The identity of products producing novel HDA patterns can be further analysed by DNA sequence analysis. In many cases, a comparison of the resulting DNA sequence to others deposited in the DNA sequence databases should allow one to identify the source of the bloodmeal unambiguously. This approach has proved useful in the identification of meats in agricultural

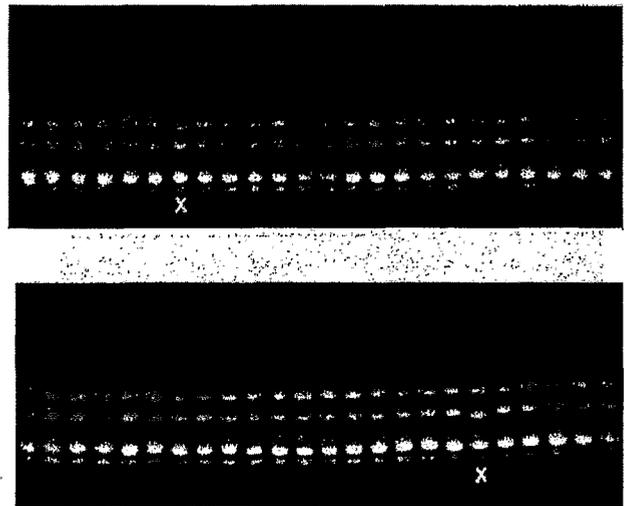


Fig. 4. Intraspecific variation of human *cytB* PCR products as detected by HDA carried out as described in Materials and Methods. Lanes marked with an X indicate individuals with HDA pattern that differed from the most common allele (samples in unmarked lanes).

studies (Bartlett & Davidson, 1992; Forrest & Carnegie, 1994). If a complete match to the unknown DNA sequence is not present in the databases, the sequence data may be subjected to phylogenetic analyses. This will identify the species most closely related to the unknown sample, providing important clues to its true identity. The identity of the bloodmeal can then be confirmed by obtaining a small DNA sample from the most likely hosts, as inferred from the phylogenetic analysis. Once a given bloodmeal is identified, the PCR product can be used to generate a new reference sample for the PCR HDA.

HDA is a very sensitive method of detecting minor sequence differences (Tang & Unnasch, 1995), so the assay could be compromised by intraspecific polymorphisms. To minimize the likelihood of this occurring, the *cytB* gene was targeted in the PCR-HDA, because it represents one of the more conserved portions of the mitochondrial genome (Cann *et al.*, 1984). We also examined the degree of intraspecific polymorphism within the *cytB* fragment in four species, as detected by the PCR HDA assay. We found no intraspecific polymorphisms detectable by HDA in the small number of pigs, domestic cattle and water buffalo samples tested. Furthermore, among >350 humans, 96% were found to contain a single *cytB* allele as determined by HDA. In the remaining 4%, some intraspecific variation was detected, but these resulted in very minor shifts in the mobility of the heteroduplex products, insufficient to preclude the correct identification of the product as human derived. Thus, the HDA detected a limited level of intraspecific heterogeneity that does not compromise the assay itself.

Some segments of the mitochondrial genome of humans do contain significant levels of intraspecific variation. This is particularly true of the control or D loop domain, which is sufficiently hypervariable to be useful for the identification of

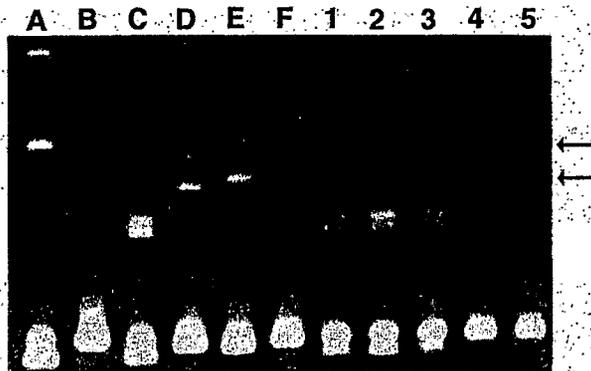


Fig. 5. Identification of bloodmeals by *cytB* PCR-HDA in wild-caught tsetse and experimentally fed blackflies—PCR amplification and HDA were carried out as described in Materials and Methods, using the water buffalo PCR product as a heteroduplex driver. Lanes A–E represent standards derived from known species. Lane A=chicken; lane B=water buffalo; lane C=domestic pig; lane D=human; lane E=chimpanzee. Lanes 1–5 represent PCR products derived from bloodmeals. Lanes 1–3 represent bloodmeals from three wild-caught tsetse flies, and lanes 4 and 5 represent bloodmeals from *S. damnosum s.l.* experimentally fed on a human volunteer. Arrows highlight the position of the bands seen in the *Simulium* bloodmeals.

individual humans in forensic studies (Stoneking *et al.*, 1991; Alonso *et al.*, 1996; Butler & Levin, 1998). The demonstration that it is possible to amplify a portion of the *cytB* gene from human-derived bloodmeals suggests that it may be possible to amplify more variable segments of the human mitochondrial genome from the bloodmeal as well. If this is the case, it may be possible to assign the source of a given bloodmeal to a particular individual, or family group. This may be useful in microepidemiological studies addressing the dispersion of haematophagous insects following bloodmeal uptake under natural conditions.

As a first test of the PCR-HDA to identify bloodmeals from field-collected insects, we amplified and characterized the DNA present in the bloodmeals of three wild-caught tsetse flies. Each contained blood identified by HDA as derived from domestic pig, confirmed by direct sequence analysis. This finding was in accordance with previous studies employing an ELISA-based assay to identify bloodmeals of *G. palpalis* (Staaik *et al.*, 1986). In this study, the majority of bloodmeals from *G. palpalis* collected in the Ivory Coast were classified as derived from pigs. Our results support the conclusion from this previous study that pigs are a common host of *G. palpalis* under natural conditions.

Results presented above indicate that a bloodmeal is detectable using the *cytB* PCR assay for a period of up to 72 h following ingestion by *S. damnosum s.l.*, sufficient to allow the PCR-based assay to be used to study the feeding preferences of wild-caught *Simulium* females. Previous studies have shown that a large proportion of *S. damnosum s.l.* females oviposit and begin searching for the next bloodmeal within 72 h after the previous bloodmeal (Thompson, 1976). The period during

which we were able to identify the bloodmeal in African *S. damnosum s.l.* was approximately 40 h less than for the ELISA method applied to North American blackflies (Hunter & Bayly, 1991). This may be attributed to the warmer temperature (25–38°C) for Afrotropical blackflies than for those in North America (9–22°C). Whereas the ELISA detects antigens that make up the bulk of the bloodmeal, the PCR relies on detection of a component (host genomic DNA) present in only a small proportion of cells in the bloodmeal. Further comparative tests are needed, at a range of controlled temperatures, to ascertain whether the antigen target of the ELISA does persist for longer than the DNA target of the PCR HDA in bloodmeals of haematophagous arthropods.

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References

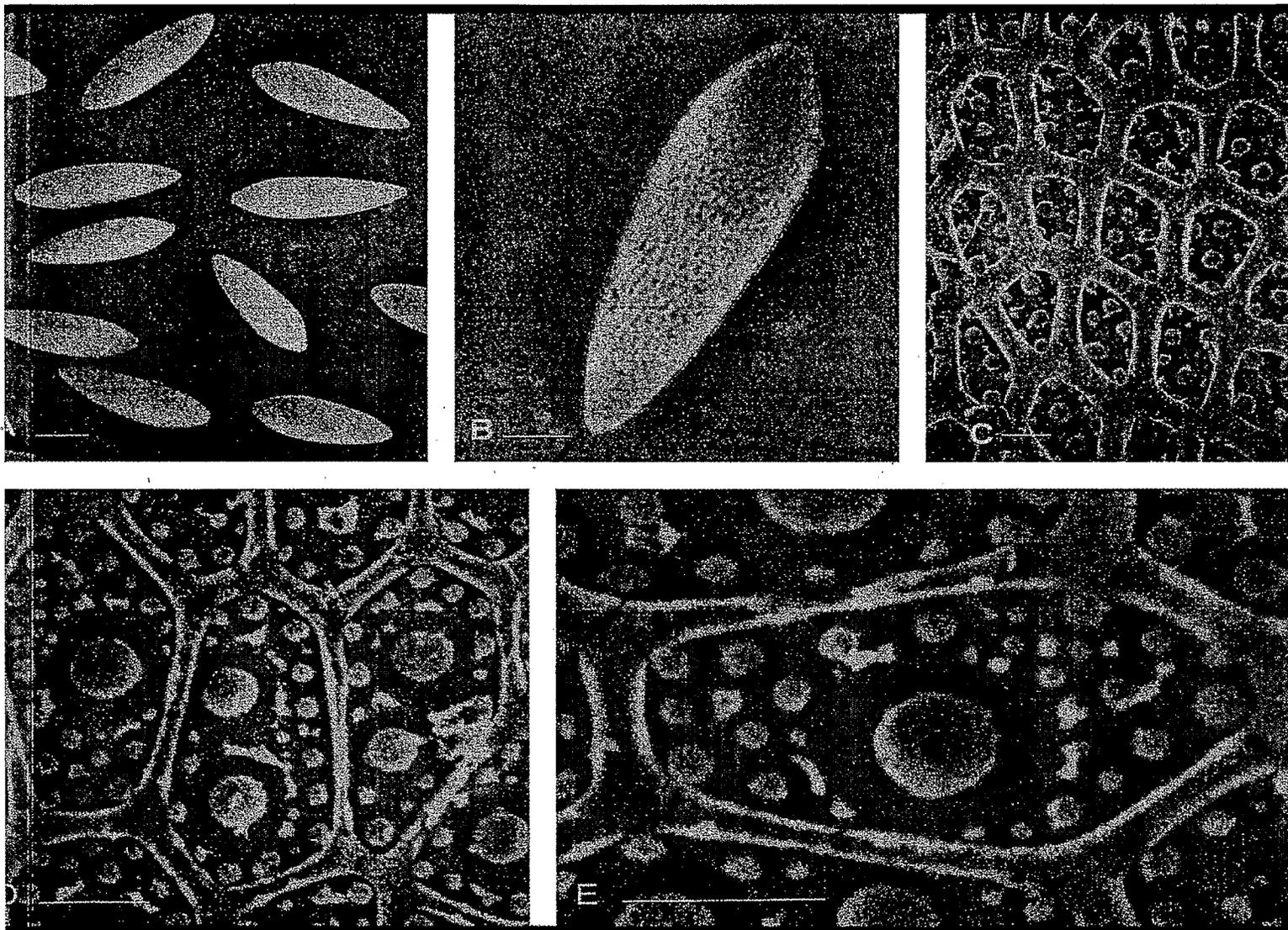
- Alonso, A., Martin, P., Albarran, C., Garcia, O. & Sancho, M. (1996) Rapid detection of sequence polymorphisms in the human mitochondrial DNA control region by polymerase chain reaction and single-strand conformation analysis in mutation detection enhancement gels. *Electrophoresis*, **17**, 1299–1301.
- Bartlett, S.E. & Davidson, W.S. (1992) FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. *BioTechniques*, **12**, 408–411.
- Butler, J.M. & Levin, B.C. (1998) Forensic applications of mitochondrial DNA. *Trends in Biotechnology*, **16**, 158–162.
- Cann, R.L., Brown, W.M. & Wilson, A.C. (1984) Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. *Genetics*, **106**, 479–499.
- Chow, E., Wirtz, R.A. & Scott, T.W. (1993) Identification of blood meals in *Aedes aegypti* by antibody sandwich enzyme-linked immunosorbent assay. *Journal of the American Mosquito Control Association*, **9**, 196–205.
- Delwart, E.L., Herrings, B., Rodrigo, A.G. & Mullins, J.I. (1995) Genetic subtyping of human immunodeficiency virus using a heteroduplex mobility assay. *PCR Methods and Applications*, **4**, S202–216.
- Delwart, E.L., Sheppard, H.W., Walker, B.D. & Mullins, J.I. (1994) Human immunodeficiency virus type 1 evolution *in vivo* tracked by DNA heteroduplex mobility assays. *Journal of Virology*, **68**, 6672–6683.
- Forrest, A.R.R. & Carnegie, P.R. (1994) Identification of gourmet meat using FINS (forensically informative nucleotide sequencing). *BioTechniques*, **17**, 24–26.
- Gokool, S., Curtis, C.F. & Smith, D.F. (1993) Analysis of mosquito bloodmeals by DNA profiling. *Medical and Veterinary Entomology*, **7**, 208–15.
- Hunter, F.F. & Bayly, R. (1991) ELISA for identification of blood

- meal source in black flies (Diptera: Simuliidae). *Journal of Medical Entomology*, **28**, 527-532.
- Kirstein, F. & Gray, J.S. (1996) A molecular marker for the identification of the zoonotic reservoirs of Lyme borreliosis by analysis of the blood meal in its European vector *Ixodes ricinus*. *Applied and Environmental Microbiology*, **62**, 4060-4065.
- Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X. & Wilson, A.C. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science USA*, **86**, 6196-6200.
- Raybould, J.N., Barro, T., Sawadogo, R. & Mordzifa, F. (1982) A new simple technique for rearing F1 progeny from females of the *Simulium damnosum* Theobald complex. *Tropical Medicine and Parasitology*, **33**, 87-93.
- Staak, C., Allmang, U.-K. & Mehltz, D. (1981) The complement fixation test for the species identification of bloodmeals from tsetse flies. *Tropical Medicine and Parasitology*, **32**, 97-98.
- Staak, C., Kampe, U. & Korčowski, G. (1986) Species identification of blood-meals from tsetse flies (Glossinidae): results, 1979-85. *Tropical Medicine and Parasitology*, **37**, 59-60.
- Steiner, J.J., Poklemba, C.J., Fjellstrom, R.G. & Elliott, L.F. (1995) A rapid one-tube genomic DNA extraction process for PCR and RAPD analyses. *Nucleic Acids Research*, **23**, 2569-2570.
- Stoneking, M., Hedgecock, D., Higuchi, R.G., Vigilant, L. & Erlich, H.A. (1991) Population variation of human mtDNA control region sequences detected by enzymatic amplification and sequence-specific oligonucleotide probes. *American Journal of Human Genetics*, **48**, 370-382.
- Tang, J., Toe, L., Back, C., Zimmerman, P.A., Pruess, K. & Unnasch, T.R. (1995) The *Simulium damnosum* species complex: phylogenetic analysis and molecular identification based upon mitochondrially encoded gene sequences. *Insect Molecular Biology*, **4**, 79-88.
- Tang, J. & Unnasch, T.R. (1995) Discriminating PCR artifacts using DHDA (directed heteroduplex analysis). *BioTechniques*, **19**, 902-905.
- Tang, J. & Unnasch, T.R. (1997) Heteroduplex analysis in medical entomology: a rapid and sensitive sequence-based tool for population and phylogenetic studies. *Parasitology Today*, **13**, 271-274.
- Thompson, B.H. (1976) The intervals between the blood-meals of man-biting *Simulium damnosum* (Diptera: Simuliidae). *Annals of Tropical Medicine and Parasitology*, **70**, 329-41.
- Zimmerman, P.A., Carrington, M.N. & Nutman, T.B. (1993) Exploiting structural differences among heteroduplex molecules to simplify genotyping the DQA1 and DQB1 alleles in human lymphocyte typing. *Nucleic Acids Research*, **21**, 4541-4547.
- Zimmerman, P.A., Shapiro, M., Tang, J., Nutman, T.B. & Unnasch, T.R. (1995) Optimizing probe selection in directed heteroduplex analysis using HDProbe 1.1. *BioTechniques*, **19**, 972-977.

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