Identification of blood meal sources of *Lutzomyia longipalpis* using polymerase chain reaction-restriction fragment length polymorphism analysis of the cytochrome B gene

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An analysis of the dietary content of haematophagous insects can provide important information about the transmission networks of certain zoonoses. The present study evaluated the potential of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the mitochondrial cytochrome B (cytb) *gene to differentiate between vertebrate species that were identified as possible sources of sandfly meals. The complete* cytb *gene sequences of 11 vertebrate species available in the National Center for Biotechnology Information database were digested with* Aci I, Alu I, Hae III *and* Rsa I *restriction enzymes* in silico *using Restriction Mapper software. The* cytb *gene fragment (358 bp) was amplified from tissue samples of vertebrate species and the dietary contents of sandflies and digested with restriction enzymes. Vertebrate species presented a restriction fragment profile that differed from that of other species, with the exception of* Canis familiaris *and* Cerdocyon thous. *The 358 bp fragment was identified in 76 sandflies. Of these, 10 were evaluated using the restriction enzymes and the food sources were predicted for four:* Homo sapiens *(1),* Bos taurus *(1) and* Equus caballus *(2). Thus, the PCR-RFLP technique could be a potential method for identifying the food sources of arthropods. However, some points must be clarified regarding the applicability of the method, such as the extent of DNA degradation through intestinal digestion, the potential for multiple sources of blood meals and the need for greater knowledge regarding intraspecific variations in mtDNA.*

Key words: blood meal analysis - cytochrome B - PCR-RFLP

Identification of the food source of arthropods allows a better understanding of the vector dynamics and transmission routes of diseases carried by vectors. It may be the best way to clarify which species are incriminated as zoonosis reservoirs and could therefore indicate better control strategies (Ribeiro 1999).

Traditionally, analysis of the dietary content of haematophagous arthropods has been carried out using immunological techniques (Mukabana et al. 2002b). However, serological methods present two limitations: (i) it is impossible to distinguish species that are phylogenetically close (Silva et al. 2001) and (ii) the analysis is restricted to one group of vertebrates because of the use of species-specific antiserum (Dias et al. 2003, Marassá et al. 2006). Studies have identified a well-preserved DNA sequence in the cytochrome B (*cytb*) gene, which is present in mitochondrial DNA and codes for an electron-transporting protein (Meece et al. 2005). This DNA sequence exhibits few intraspecies variations, but sufficient interspecies variations, thereby increasing its specificity. Thus, a large number of hosts may be analysed using universal primers and phylogenetically close species may be distinguished using a molecular test based on the *cytb* gene (Boakye et al. 1999, Chow-Shaffer et al. 2000, Lee et al. 2002, Meece et al. 2005, Steuber et al. 2005, Muturi et al. 2011, Garlapati et al. 2012, Tiwananthagorn et al. 2012, Pettersson et al. 2013).

The aim of the present study was to provide theoretical validation for using PCR-RFLP of the *cytb* gene as a technique to discriminate the blood meal source of *Lutzomyia longipalpis*. The restriction fragment profiles of some vertebrate species identified as possible sandfly food sources were analysed. It was found that the species could be differentiated according to the sizes of the restriction fragments.

The mitochondrial *cytb* gene fragments of the 11 species used in this study (*Homo sapiens* - HOSA, *Rattus norvegicus* - RANO, *Didelphis marsupialis* - DIMA, *Canis familiaris* - CAFA, *Felis catus* - FECA, *Sus domesticus* - SUDO, *Bos taurus* - BOTA, *Gallus gallus* - GAGA, *Equus caballus* - EQCA, *Cerdocyon thous* - CETH and *Pseudalopex*

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vetulus - PSVE) were amplified *in silico* using the primers BM1 (5'CCCCTCAGAATGATATTTGTCCTCA3') and BM2 (5'CCATCCAACATCTCAGCATGATGAAA3') and Basic Local Alignment Search Tool software, which is available from the National Center for Biotechnology Information (GenBank). The sequence of the expected product (358 bp) was species-specific and the *Aci I*, *Alu I*, *Hae III* and *Rsa I* restriction sites were identified using Restriction Mapper software. The restriction profiles of the fox species *P. vetulus* and *C. thous* were the only two that were not analysed because it was not possible to identify the 358 bp *cytb* fragment in these species in GenBank. Each species had a restriction fragment profile that was distinct from the others. Thus, these profiles represented a unique "fingerprint", which could be an important method for distinguishing between each species.

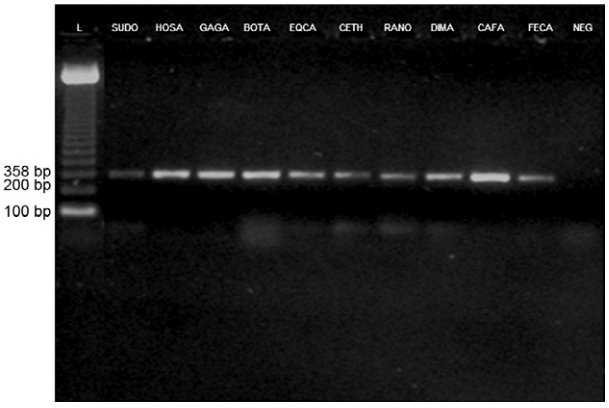

Fig. 1: electrophoresis with polymerase chain reaction on the DNA extracted from peripheral blood and tissue samples from the vertebrate species amplified using the BM1 and BM2 primers (fragment of mitochondrial *cytb* ≅ 358 bp). L: DNA Leader 100 bp; NEG: negative control.

Biological samples (peripheral blood or cellular tissue) of the vertebrate species were collected and DNA was extracted. The mitochondrial *cytb* gene fragment (358 bp) was amplified using the BM1 and BM2 primers following the protocol of Meece et al. (2005) (Fig. 1). However, PSVE presented a unique problem: the polymerase chain reaction (PCR) amplified not only the 358 bp fragment, but also a fragment of approximately 615 bp. This fox species is rare and threatened, lives mainly in the central part of Brazil and was excluded from the study (Costa & Courtenay 2003). However, the presence of these two bands allowed for the differentiation of PSVE from the other animals.

The amplified fragments were sequenced to determine the degree of conservation of the *cytb* gene. Comparing the sequenced loco-regional samples with the deposited GenBank sequences, most species presented few mismatches, with no compromised restriction sites and with degrees of similarity greater than 98% (Table I). There were no changes in the restriction sites of CAFA, HOSA, GAGA, RANO, SUDO or BOTA. However, EQCA and FECA gained *Aci* I and *Rsa* I restriction sites, respectively and DIMA lost two *Alu* I restriction sites (Table I, Supplementary data). Because the number of *cytb* sequences deposited in GenBank is limited, sequencing of this fragment in loco-regional host species becomes important for analysis using the PCR-restriction fragment length polymorphism (RFLP) technique. As *cytb* sequences continue to be deposited into these genetic databases and as the variations in mtDNA become better understood, sequencing of this fragment from loco-regional host species will be needed less frequently, thus reducing costs and increasing the applicability of the method.

TABLE I

Identification of the species that were candidates as possible food sources for sandflies

Species	Common name	Access (BLAST/NCBI)	Similarity GenBank/Regional (%)	Changes in restriction sites
Homo sapiens (HOSA)	Human	AY509658	99	0
Rattus norvegicus (RANO)	Rats	AB033713	98	0
Didelphis marsupialis (DIMA)	Opossum	DMU34665	97	-2
Canis familiaris (CAFA)	Dog	DQ309764	100	0
Felis catus (FECA)	Domestic cats	AY509646	99	+1
Sus domesticus (SUDO)	Pig	AY534296	99	0
Bos taurus (BOTA)	Cattle	AY682380	99	0
Gallus gallus (GAGA)	Hens	AY509649	100	0
Equus caballus (EQCA)	Horse	AY819736, AY819737	99	+1
Cerdocyon thous (CETH)	Crab-eating fox	AF028169	83[a]	Unparsed
Pseudalopex vetulus (PSVE)	Hoary fox	EF106996	-	Unparsed

a: the sequenced fragment of cytochrome B gene had a 99% affinity with the *Canis familiaris* from GenBank; BLAST: Basic Local Alignment Search Tool; NCBI: National Center for Biotechnology Information.

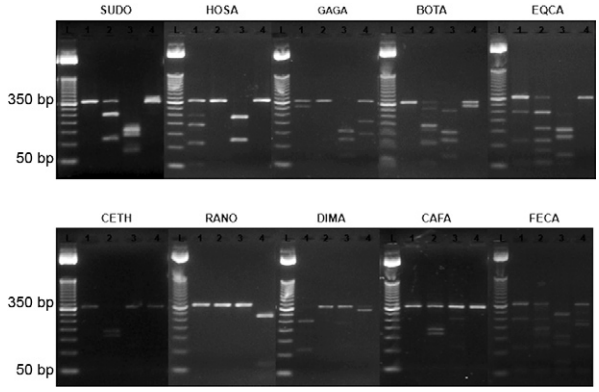

Fig. 2: polymerase chain reaction-restriction fragment length polymorphism on the DNA samples of interest amplified using the BM1 and BM2 primers and digested using *Aci I*, *Alu I*, *Hae III* and *Rsa I* (fragment of mitochondrial *cytb* ≅ 358 bp). L: DNA Leader 50 bp; 1: *Aci I*; 2: *Alu I*; 3: *Hae III*; 4: *Rsa I*.

To confirm the *in silico* analysis, the PCR products were submitted to enzymatic digestion using the *Aci I*, *Alu I*, *Hae III* and *Rsa I* enzymes. The restriction profiles of the species studied are shown in Fig. 2 and Table II. In practice, the restriction pattern of *Aci* I showed the expected fragments (244 bp and 113 bp) for DIMA, FECA and EQCA. Regarding the DNA of HOSA, a 244 bp band was found in addition to the expected fragments (189 bp, 113 bp and 55 bp). The expected restriction pattern of GAGA was observed; however, the 49 bp fragment could not be visualised because of its small size. The *Aci* I endonuclease did not cut the DNA of CAFA, BOTA, SUDO, RANO or CETH.

When the DNA samples were digested with *Alu* I enzymes, the expected restriction fragments for SUDO (242 bp and 115 bp) and BOTA (190 bp, 114 bp and 53 bp) were confirmed. However, BOTA presented an additional nonspecific fragment (304 bp). No *Alu* I restriction site

TABLE II

Restriction profiles of the species of interest using the mtDNA 358 bp fragment

Host species	Restriction enzymes (cleavage sites)			
	Aci I CC↓GC	*Alu I* AG↓CT	*Hae III* GG↓CC	*Rsa I* GT↓AC
Homo sapiens (HOSA)				
Sequenced	189; 113; 55	358	233; 124	358
NCBI (AY509658)	188; 114; 55	358	232; 125	358
Rattus novergicus (RANO)				
Sequenced	358	358	358	267; 59; 31
NCBI (AB033713)	358	358	358	267; 59; 31
Didelphos marsupialis (DIMA)				
Sequenced	244; 113	358	358	326; 31
NCBI (DMU34665)	244; 133	272; 69; 16	358	326; 31
Canis familiaris (CAFA)				
Sequenced	358	190; 167	358	358
NCBI (DQ309764)	358	190; 165	358	358
Felis catus (FECA)				
Sequenced	244; 113	190; 120; 47	272; 74; 11	214; 119; 24
NCBI (AY509646)	243; 114	189; 120; 48	273; 73; 11	213; 144
Sus domesticus (SUDO)				
Sequenced	358	242; 115	153; 130; 74	358
NCBI (AY534296)	358	242; 115	153; 130; 74	358
Bus taurus (BOTA)				
Sequenced	358	190; 114; 53	159; 124; 74	322; 31; 4
NCBI (AY682380)	358	190; 169	159; 126; 74	322; 33; 4
Gallus gallus (GAGA)				
Sequenced	308; 49	358	159; 124; 74	208; 149
NCBI (AY509649)	309; 48	358	159; 125; 73	209; 148
Equus caballus (EQCA)				
Sequenced	244; 113	160; 85; 59; 53	159; 124; 74	358
NCBI (AY819736)	358	160; 84; 59; 54	159; 125; 73	358
Cerdocyon thous (CETH)				
Sequenced	358	190; 167	358	358
NCBI (AF028169)[a]	-	-	-	-

a: the prime BM1 did not recognise a sequence of cytochrome B gene; NCBI: National Center for Biotechnology Information.

was found for DIMA, RANO, GAGA or HOSA. In addition to the expected fragments for EQCA and FECA, unexpected fragments were also observed: 242 bp and 115 bp for EQCA and 237 bp for FECA. CETH and CAFA presented bands of 190 and 167 bp, as expected (Fig. 2, Table II). As reported by other authors, nonspecific bands were also visualised in the present study (Zhang & Hewit 1996, Partis et al. 2000, Steuber et al. 2005). One explanation for this phenomenon could be the co-amplification of *cytb* pseudogenes. Pseudogenes are non-functional copies of mtDNA fragments present in nuclear DNA. However, when the expected fragments are also viewed, identification of the species is not impaired.

Digestion with the *Hae* III endonuclease allowed differentiation between several species of vertebrates. GAGA and EQCA presented the same pattern of bands (159 bp, 124 bp and 74 bp); however, BOTA presented an extra fragment (290 bp). SUDO (130 bp, 153 bp and 74 bp) and HOSA (233 bp and 124 bp) showed the expected patterns. No *Hae* III restriction site was found for CAFA, CETH, DIMA or RANO. FECA presented both nonspecific and expected bands (Fig. 2, Table II).

Digestion with the *Rsa* I enzyme made it possible to differentiate CETH, RANO, GAGA and SUDO from the other host species. The restriction pattern for GAGA was as expected (208 bp and 149 bp). BOTA and DIMA presented single fragments of 322 bp and 326 bp, respectively. Fragments smaller than 31 bp were not visualised. *Rsa* I did not cut DNA extracted from HOSA, EQCA, SUDO, CETH or CAFA (Fig. 2, Table II).

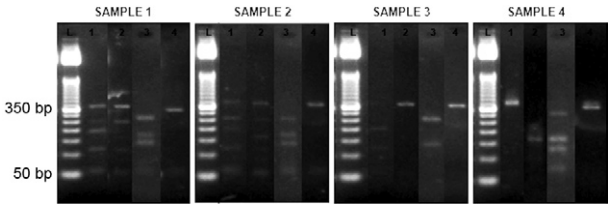

Fig. 3: polymerase chain reaction-restriction fragment length polymorphism on the DNA samples from sandflies amplified using the BM1 and BM2 primers and digested using *Aci I*, *Alu I*, *Hae III* and *Rsa I* (fragment of mitochondrial *cytb* ≅ 358 bp). L: DNA Leader 50 bp; 1: *Aci I*; 2: *Alu I*; 3: *Hae III*; 4: *Rsa I*.

The enzymes *Aci I*, *Alu I*, *Hae III* and *Rsa I* were sufficient for the differentiation of the species of interest, with the exception of CAFA and CETH, which presented the same restriction profile. A fifth enzyme or other genetic marker could be used to differentiate these two species. This differentiation is of great epidemiological interest because foxes and dogs share an important role as reservoirs of visceral leishmaniasis. As foxes have periurban habitats, this identification method could confirm whether foxes maintain the urban cycle (Costa & Vieira 2001, Silva et al. 2001).

To verify whether PCR-RFLP of the *cytb* gene can be used to evaluate the blood meal source of sandflies, a total of 80 female specimens of *Lu. longipalpis* were caught in domestic and peridomestic environments using two electrically powered CDC light traps. DNA was extracted from the intestinal contents of sandflies and PCR amplification was performed using BM1 and BM2 primers. The 358 bp fragment of *cytb* was identified in 76 samples. Most of the sandflies had blood in their digestive tubes. Initially, this finding enabled analysis by means of sequencing or PCR-RFLP. Of the 76 samples, 10 were chosen randomly and their PCR products were individually digested using four restriction enzymes (*Aci I*, *Alu I*, *Hae III* and *Rsa I*). In four out of the 10 sandflies, the food sources were assumed (Fig. 3, Table III). The food sources of samples 1 and 2 were believed to be EQCA. The probable food sources of samples 3 and 4 were HOSA and BOTA, respectively. Some fragments could not be identified because of their size or due to the small amount of DNA present. It is possible that the low quantity DNA resulted in weak or even missing band signals, making it difficult to analyse the fragment profile. Theoretically, this technique could become less accurate if DNA was degraded via intestinal digestion (Mukabana et al. 2002a). A detailed analysis of sandflies in the laboratory, using a known food source and performing measurements at specific time points, could resolve this issue.

Therefore, the PCR-RFLP technique has the potential to be useful for phlebotomine blood meal source identification. However, some points must be clarified regarding the applicability of the method, such as the extent of DNA degradation through intestinal digestion, the potential for multiple sources of blood meals and the need for greater knowledge of intraspecific mtDNA variations.

TABLE III

Probable restriction profiles of the sandflies using the mtDNA 358 bp fragment

	Restriction enzymes (cleavage sites)			
Sandflies	*Aci I* CC↓GC	*Alu I* AG↓CT	*Hae III* GG↓CC	*Rsa I* GT↓AC
Sample 1	244; 180; 133; 55	242; 159; 113; 55	159; 124	358
Sample 2	244; 180; 133; 55	242; 159; 113; 55	159; 124	358
Sample 3	189; 113	358	233; 124	358
Sample 4	358	190; 114	290; 159; 124; 74	322

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