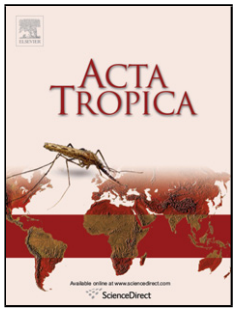

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Identification of blood meals in field captured sand flies by a PCR-RFLP approach based on cytochrome *b* gene

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Abstract

Leishmaniasis is a vector-borne disease transmitted byphlebotominesand flies. Information about blood meal preferences in sand flies is essential to understand the epidemiology of the diseaseto adopt control measures.In previous studies, a polymerase chain reaction (PCR) of 359 bp fragment of the conserved gene cytochrome *b* (*cyt b*) and further sequencing were applied in the study of blood meal sources in sand flies collected in the area of a leishmaniasis outbreak insouthwest Madrid, Spain, providing significant information about blood meal preferences in the focus.

In this work, aPCR-restriction fragment length polymorphism(RFLP)targeting afragment of 359 bpof vertebrate *cytb*gene was developed. Restriction

endonucleases*Hae*III and *Hinf*I generated specific patterns consistent with the blood meal sources found in sandflies. The protocol has been validatedwithtwenty sixengorged females collected in the field with CDC traps.Blood meals from nine vertebrateswere identified based on PCR-*cytb* and sequencing -human, dog, cat, horse, hare, rabbit, sheep, goat and chicken - and mixed blood meals (sheep/human;sheep/goat) – and successfully distinguished byPCR-RFLP. Therefore, thisapproachis anefficient and reliable alternative method to be applied in entomological surveys.

Keywords: PCR-RFLP, sand fly, blood meal, cytochrome *b*

1. Introduction

Leishmaniasis is a vector-borne disease caused by a trypanosomatid protozoan of the genus *Leishmania* (WHO 2010). In Spain it is caused by *Leishmaniainfantum*, using the vectors*Phlebotomusperniciosus* and *Phlebotomusariasi*(Rioux et al. 1986). The dogis the main reservoir, although it has been demonstrated that other wild mammalscan play an important role in the maintenance and transmission of the parasite(Molina et al. 2012, Del Río et al. 2013, Ruiz-Fons et al. 2013, Jiménez et al. 2014).

Although leishmaniasis in Spain is considered hypoendemic, some regions are more affected,like the Mediterranean coast and the center of Spain.Moreover, since 2010 an important increase of the disease in urban areas of southwest Madrid has been detected. Actually, one of the areas studied in this study belongs to this affected area, where the mean incidence rate reached 44.55 cases per100,000 inhabitants in 2013 (data provided by Autonomous Community of Madrid). In case of Mediterranean area, other studied region, Minorca (Balearic Islands), were reported an emergence of canine leishmaniasis(Gil-Prieto et al. 2011, Alcover et al. 2013, Arce et al. 2013, Ballart et al. 2013).

The knowledge of possible reservoirs implicated in an area affected with leishmaniasis is essential when deciding which control measures are needed to take. In this sense, thestudy of blood meal sources of vectors plays an essential role.Accordingly, serological techniquesas ELISA were appliedas first approachin this way(De Colmenares et al. 1995, Bongiorno et al. 2003), although these methods have been replaced by molecular tools for their better accuracy (Mukabana et al. 2002, Haouas et al. 2007, Kent 2009,Maleki-Ravasan et al. 2009). Amplification by polymerase chain reaction (PCR) and subsequent sequencingis widely used to study

bloodmeal preferences in sand flies and other vectors as mosquitoes or ticks (Kirstein and Grey 1996, Oshagi et al. 2006, Danabalan et al. 2014).Thus, different molecular targets like cytochrome *b* (*cytb*), cytochrome oxidase I (COI) and prepronociceptin (PNOC) are being used to detect the origin of blood meals (Kocher et al. 1989, Haoaus et al. 2007, Abassi et al. 2009).On the other hand, other techniques as PCR- restriction fragment length polymorphism (RFLP)or PCR-reverse-line blotting (RLB) have been developed in order to study blood meal preferences in mosquitoes, tsetse flies and sand flies(Osaghi et al. 2006, Steuber et al. 2005, Maleki-Ravasan et al. 2009, Quaresma et al. 2012, Soares et al. 2014, Gebresilassie et al. 2015).

In previous studies, PCR of a 359 bp fragment of the conserved gene *cytb* and further sequencing were applied in the study of blood meal sources in sand flies collected in the area of a leishmaniasis outbreak occurring in southwest Madrid region, Spain. This method provided significant information about the role that hares and rabbits are playing as reservoirs, in the focus (Jiménez et al. 2013, 2014).

The aim of this work was to develop a PCR-RFLP method targeting the fragment of 359 bp of vertebrate *cytb* gene. The protocol was validated in twenty sixblood fed female sand flies collected in the field with CDC traps in three separate entomological surveys carried out during 2012, 2013 and 2014 in three different regions of Spain. Blood meal preferences in all sand flies were studied at first by PCR of the 359 bp fragment of *cytb* following sequencing.

Thedeveloped *cytb*PCR-RFLP proved to be an effective and reliable approach in the identification of blood meals in sand flies,alternative to the PCR and sequencing method, to be used in entomological surveys.

2. Material and methods

2.1. Sand flies and DNA extraction

Female sand flies used in this study were collected with CDC traps in three separate entomological surveys carried out in the transmission period of 2012, 2013 and 2014 in three different regions from Spain: Fuenlabrada (southwest Madrid, center of Spain, n=17), Girona (northeast Catalonia, northeast of Spain, n= 7) and Minorca (Balearic Islands, n=2) (Table 1). In all cases sand flies were stored in ethanol 70º at 4ºC until processed.

Sand flies were washed individually in sterile distilled water using ELISA plates to eliminate ethanol. After that, wings and legs were removed, and head and genitalia were cleared and mounted in Hoyer mediumfor later morphological identification under the microscope.

DNA of sand flies was obtained from thorax and abdomen using the DNeasy® Blood & Tissue Extraction Kit (Qiagen, Hilden, Germany). Quantification and purity of the DNA was determined by spectrophotometry with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). In all cases DNA samples were stored at -20 ºC until use.

2.2. PCR-cytband sequence analysis

Blood meal identification in sand flies was conducted by the amplification of a fragment of 359 bp of vertebrate*cytb* gene followed by sequencing as described before (Jiménez et al. 2013, 2014).Multiple sequence alignment was obtained by Clustal W method in Megalign program (DNASTAR, Lasergen v7.1®, Madison, WI) with homologous sequencesof the 359 bp*cytb* gene fragment of different species of

vertebrates found in sand flies blood meals obtained using BLAST (http://blast.ncbi.nlm.nih.gov/) and retrieved from Genbank^{TM}. The following sequences were used: *Canisfamiliaris* (DQ309764.1); *Feliscatus* (AY509646.1); *Homo sapiens* (KJ154500.1, KM102050.1, KM213520.1); *Lepusgranatensis* (JF299042.1) and *L. timidus* (HM233015.1); *Oryctolaguscuniculus* (HG810791.1, HQ596486.1, FM205927.1); *Caprahircus* (FM205715.1, GU120393.1) *Equuscaballus* (KF038165.1); *Gallusgallus* (KF981434.1) and *Ovisaries*(AJ971339.1, JX567811.1, KF977846.1). The predicted endonuclease restriction sites *Hae*III and *Hinf*I were analyzed with the bioinformatics program Seqbuilder (DNASTAR, Lasergen v7.1®, Madison, WI)(Fig. 1).

2.3. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism of cytb (PCR-RFLPcyt b)

Amplification of a fragment of359 bp of *cytb*genewas performed as defined before withfew modifications (Jiménez et al. 2013, 2014). Degenerated primers: cyt_bb1 (5´-CCA TCM AAC ATY TCA DCA TGA TGA AA-3´) and cyt_bb2 (5´-GCH CCT CAG AAT GAY ATT TGK CCT CA-3)were used (Svobodova et al. 2009). PCR was carried out with 70 ng of DNA in a final volume of 25 µl: 1x Buffer (Roche®, Basel, Switzerland), 1.5 mM $MgCl_2$(Roche®, Basel, Switzerland), 100 µM dNTPs mixture (10 mM, Roche®, Basel, Switzerland), AmpliTaqGold (Roche®, Basel, Switzerland), 1 µl of BSA DNAse Free (20 mg/ml, Roche®, Basel, Switzerland). Primers were used in a concentration of 0.5 µM. PCR amplification was performed as follows: one cycle of 9 minutes at 95ºC, followed by 40 cycles consisting of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and elongation at 72ºC for 45 seconds; final elongation was at 72ºC for 10 minutes (ThermocycleGeneAmp ® PCR

system 2700, Applied Biosystems, Foster, CA).Subsequently, 25 µL of each PCR product was digested with 3U of *Hae*III and 5U of *Hinf*I at 37ºC overnight in 30 µl of reaction volume according to the manufacturer's protocols (New England Biolabs, UK). The digested PCR products were then fractionated in 3% MS-8 agarose (Conda®, Spain) gel stained with Pronasafe Nucleic Acid Staining Solution (10 mg/ml) (Conda®, Spain) and specific band patterns were observed under UV light exposure.

3. Resultsand Discussion

A total of twenty-six blood fed sand flies captured in three different entomological surveys performed in different locations during the transmission period of 2012, 2013 and 2014 were selected for this study. Of these, seventeen fed female sand flies were captured in the active human leishmaniasis focus of Fuenlabrada (Madrid). Moreover, seven sand flies from Girona (Catalonia) and two from Minorca (Balearic Islands) were analyzed (Table 1).

Firstly, the analysis of blood preferences was carried out by the amplification of a fragment of 359bp of vertebrate *cytb* gene, further sequencing, and subsequent comparison with homologous sequences deposited in the GenBank™using BLAST (http://blast.ncbi.nlm.nih.gov/). Regarding to the sand flies from Madrid, all were identified as *P. perniciosus*. Analysis of blood feeding preferences revealedthat sand flies mainly fed on rabbit(n=9). Other sources of vertebrates found were hare (n=4), human (n=2), cat (n=1) and dog (n=1) (Table 1).

Moreover, sand flies from Girona were identified as *P. perniciosus*(n=2) and *P. ariasi*(n=3). From these,one *P. perniciosus*was fed on horse and two *P. ariasi*on

sheep. Additionally,two mixed blood meals- sheep/humanand sheep/goat- were detected in both*P. perniciosus*(n=1) and*P. ariasi*(n=1), respectively. In the remaining two sand flies from Girona(species not identified, there were no genitalia) mixed blood meals -sheep/goat-were detected.The two *P. perniciosus*from Minorca were fed on chicken and goat,respectively (Table 1).

Analysis of multiple sequence alignment by CLUSTAL W method of homologous sequences of 359 bp of *cytb* was carried out searching for specific restriction sites in each sequence for a RFLP approach.Restriction endonucleases*Hae*IIIand*Hinf*I were able to distinguish a differential pattern offragmentsin thetargeted species according with the results obtained by the bioinformatic program Seqbuilder(Fig. 1). With the aim to confirm these bioinformatic analysis, PCR products of 359 bp*cyt b*gene obtained from fed sand flies previously described were digested with restriction endonucleases *Hae*III and *Hinf*I.

The RFLP band pattern produced by these two restriction enzymes used in combination is able to discriminate among the 359 bpcyt*b* fragments of all the vertebrates analyzed (Fig. 2 and 3). In addition, the band patternobtained using *Hae*III and *Hinf*I shows differences in the 359 bp*cytb* fragmentevenamongclosely related species of hares as*L. granatensis*and*L.timidus*, which appear in Spain as an introgression of the *cytb* gene (Alves et al. 2003, Melo-Ferreira et al. 2012) (Fig. 2A and 3A). In this way, hares have been demonstrated to be an important reservoir in the active focus of leishmaniasis in southwest of Madrid. In this sense, this PCR-RFLP *cyt b* method allows to differentiate this reservoir from the others found in the focus (Molina et al. 2012, Jiménez et al. 2013).

Moreover,digestion with restriction enzyme *Hae*III was not able to discriminate among two vertebrate sequences (Fig. 2D), this is the case of *E. caballus*

(KF038165.1, lane 3)and *G. gallus*(KF981434.1, lane 4) giving rise to 20, 75, 105 and 159 bpband pattern. On the other hand,*Hinf*I enzyme solves this matchby providing a different band pattern enable to distinguish these species (Fig. 3C).Otherwise,*Hinf*I could not differentiate between sequences (Fig. 3A) as *L. granatensis*(JF299042.1, lane 6) and *O. cuniculus*(HQ596486.1, lane 1)appear with an identical band pattern of 25, 125 and 162 bp; or between*L. timidus* (HM233015.1, lane 8) and *O. cuniculus* (HG810791.1, lanes 2, 4, 7; FM205927.1, lane 3),which match in a band pattern of 125 and 234 bp (Table 1 and Fig. 3A).In these cases, *Hae*III is able to distinguish these species (Fig. 2A). Consequently, it is necessary to use the two restriction enzymes in order to obtain conclusive results.

Unexpected bands appear in the gel visualization in some specimens. Partial digestion or contamination seems unlikely; consequently the most probable explanation of these bands could be the co-amplification of *cytb* pseudogenes, as reported before (Meyer et al. 1995 and Partis et al. 2000).

Moreover, when analyzingblood meals of female sand flies from Fuenlabrada fed on rabbits and hares, we found some nucleotide polymorphism between sequences of the same species. Some of these differences don't affect the restriction sitesand in BLAST analysis homologous sequences JF299042.1 and HG810791.1 matching *cytb* sequences corresponding to *L. granatensis* and O*. cuniculus* present the maximum similarity situated between 97-99% ([b] labelled in Table 1). On the other hand, some nucleotide differencesaffect therestriction sites like in rabbit and goat sequences. Thus, 359 bp*cyt* b sequences that match with Genbank™homologous sequences*O. cuniculus* (HG810791.1)and *C. hircus* (GU120393.1) present one more restriction sitewith *Hae*III, located at position nt=76and nt=235, respectively ([c] labelled in Table 1, Fig. 1). In the case of restriction enzyme *Hinf*I,the homologous

sequencein Genbank™HQ596486.1 shows one more restriction site at position nt=165, which is not present in the other sequences of rabbits([d] labelled in Table 1, Fig. 1). In case of homologous Genbank™ sequence DQ309764.1, corresponding to *C. familiaris*, *Hinf*I cuts in two sites according to bioinformatics study, however gel visualization did not show any band pattern (Fig. 2D and 3A). These differences are visible in the band fragment pattern and might possibly be due to polymorphism within the species. This intra-specific variation in *cytb* gene has been previously reported (Meyer et al. 1995, Branco et al. 2000).

Study of mixed blood meals by sequencing can be difficult in some cases due to the apparition of background peaks that complicate the identification of possiblemixed blood meals. Similarly, although the ELISA and PCR-RLB techniques may allow identifying mixed blood meals by testing different antigens and probes, respectively (De Colmenares et al. 1995, Rossi et al. 2008, Abassi et al. 2009)both methods are more complex than PCR-RFLP. Moreover, in ELISA based methods frequently cross-reactionswere observed between close species (De Colmenares et al. 1995). On the other hand, in PCR-RLB the design and application of species-specific probes fora large number of different hosts is difficult to optimize and several problems with cross-hybridization occur (Abassi et al. 2009).

As an alternative, PCR-RFLP methodology shows a simple analyzable band pattern. In this study, mixed blood meals from four sand flies were distinguishedby *cytb*-RFLP protocol, all of them from Girona (Table 1). Both restriction enzymes, *Hae*III and *Hinf*I, generate a differential pattern that allows distinguishing the mixed blood meal from human and sheep, (75, 125, 159 / 20, 105, 234 bp) and (64, 295 / 162, 197bp), respectively found in *P. perniciosus*(Fig. 2B and Fig 3A, 3B). In case ofgoat/sheepmixed bloodmeals found in one *P. ariasi* and two unclassified sand flies,

the restriction enzyme study at first indicated that only *Hinf*I was able to discriminate between the two species (Fig. 1 and Table 1), although band pattern visualization after digestion with both enzymes showed that the two restriction enzymes could differentiate this mixed blood meal (Fig. 2C and Fig. 3B). This discrepancy among the bioinformatic study and band pattern visualization may possibly be caused by polymorphism in the nucleotide sequence, as explained before.

Accordingly, the *cytb*-RFLP presented here supportsthe results previously obtained by PCR following by sequencingof the 359 bp fragment of *cytb*(Jiménez et al. 2013, Jiménez et al. 2014). Moreover, this method is less time consuming and could be an alternative to sequencing.

In conclusion, this PCR-RFLP*cytb* method showsto behighly effectivefor the analysis ofblood meal preferencesand the differentiationof mixedblood meals in phlebotomine sand flies, caught in Spain. Although, this method should be evaluated and validated by investigators in the specific regions of study in order to apply it in the identification of sand fly blood meal preferences.

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Conflict of interest

Theauthors declare no conflicts of interest.

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Table 1.Sand fly description and corresponding blood meal identification by PCR-sequencing and *cytb*-RFLP.

Sandflyidentification	Sand fly origin/ year	PCR cyt*b*-sequencing	Genbank™ homologoussequence (% Similarity)	cyt*b*-RFLP	
				***Hae*III (bp)**	***Hinf*I (bp)**
P. perniciosus	Madrid/ 2014	*Oryctolaguscuniculus*	HG810791.1[b, c] (97%)	30, 45, 131, 153	125, 234
P. perniciosus	Madrid/ 2014	*Oryctolaguscuniculus*	HG810791.1[b, c] (98%)	30, 45, 131, 153	125, 234
P. perniciosus	Madrid/ 2014	*Oryctolaguscuniculus*	HG810791.1[b, c] (99%)	30, 45, 131, 153	125, 234
P. perniciosus*	Madrid/ 2013	*Oryctolaguscuniculus*	HG810791.1[b] (99%)	30, 45, 131, 153	125, 234
P. perniciosus*	Madrid/ 2013	*Oryctolaguscuniculus*	HG810791.1[b] (99%)	30, 45, 131, 153	125, 234
P. perniciosus*	Madrid/ 2013	*Oryctolaguscuniculus*	HG810791.1[b, c] (99%)	30, 45, 131, 153	125, 234
P. perniciosus*	Madrid/ 2013	*Oryctolaguscuniculus*	HG810791.1[b, c] (99%)	30, 45, 131, 153	125, 234
P. perniciosus*	Madrid/ 2013	*Oryctolaguscuniculus*	HQ596486.1[d] (92%)	45, 131, 183	72, 125, 162
P. perniciosus*	Madrid/ 2013	*Oryctolaguscuniculus*	FM205927.1 (96%)	45, 131, 183	125, 234
P. perniciosus	Madrid/ 2014	*Lepusgranatensis*	JF299042.1[b] (98%)	131, 228	72, 125, 162
P. perniciosus	Madrid/ 2014	*Lepusgranatensis*	JF299042.1[b] (98%)	131, 228	72, 125, 162
P. perniciosus*	Madrid/ 2013	*Lepusgranatensis*	JF299042.1[b] (99%)	131, 228	72, 125, 162
P. perniciosus*	Madrid/ 2013	*Lepustimidus*	HM233015.1 (99%)	75, 284	125, 234
P. perniciosus	Madrid/ 2014	*Homo sapiens*	KM102050.1 (99%)	20, 105, 234	162, 197
P. perniciosus*	Madrid/ 2013	*Homo sapiens*	KM213520.1 (99%)	20, 105, 234	162, 197
P. perniciosus*	Madrid/ 2013	*Feliscatus*	AY509646.1 (99%)	11, 20, 74, 254	45, 79, 117, 117
P. perniciosus*	Madrid/ 2012	*Canisfamiliaris*	DQ309764.1 (89%)	359	9, 55, 295
P. perniciosus	Girona/ 2012	*Equuscaballus*	KF038165.1 (99%)	20, 75, 105, 159	45, 80, 234
P. ariasi	Girona/ 2012	*Ovisaries*	JX567811.1 (99%)	75, 125, 159	64, 295
P. ariasi	Girona/ 2012	*Ovisaries*	KF977846.1 (99%)	75, 125, 159	64, 295
P. ariasi	Girona/ 2012	*Ovisaries/ Caprahircus*	AJ971339.1 (96%)/GU120393.1[c] (96%)	75, 125, 159	64, 295 /163,196
NI[a]	Girona/ 2012	*Ovisaries/ Caprahircus*	AJ971339.1 (96%)/GU120393.1[c] (96%)	75, 125, 159	64, 295 /163,196
NI[a]	Girona/ 2012	*Ovisaries/ Caprahircus*	AJ971339.1 (96%)/GU120393.1[c] (96%)	75, 125, 159	64, 295 /163,196
P. perniciosus	Girona/ 2012	*Ovisaries/Homo sapiens*	KF977846.1 (99%)/KJ154700.1 (97%)	75, 125, 159 / 20, 105, 234	64, 295 /163, 196
P. perniciosus	Minorca/ 2013	*Caprahircus*	FM205715.1[c] (99%)	54, 75, 230	163, 196
P. perniciosus	Minorca/ 2013	*Gallusgallus*	KF981434.1 (99%)	20, 75, 105, 159	10, 162, 187

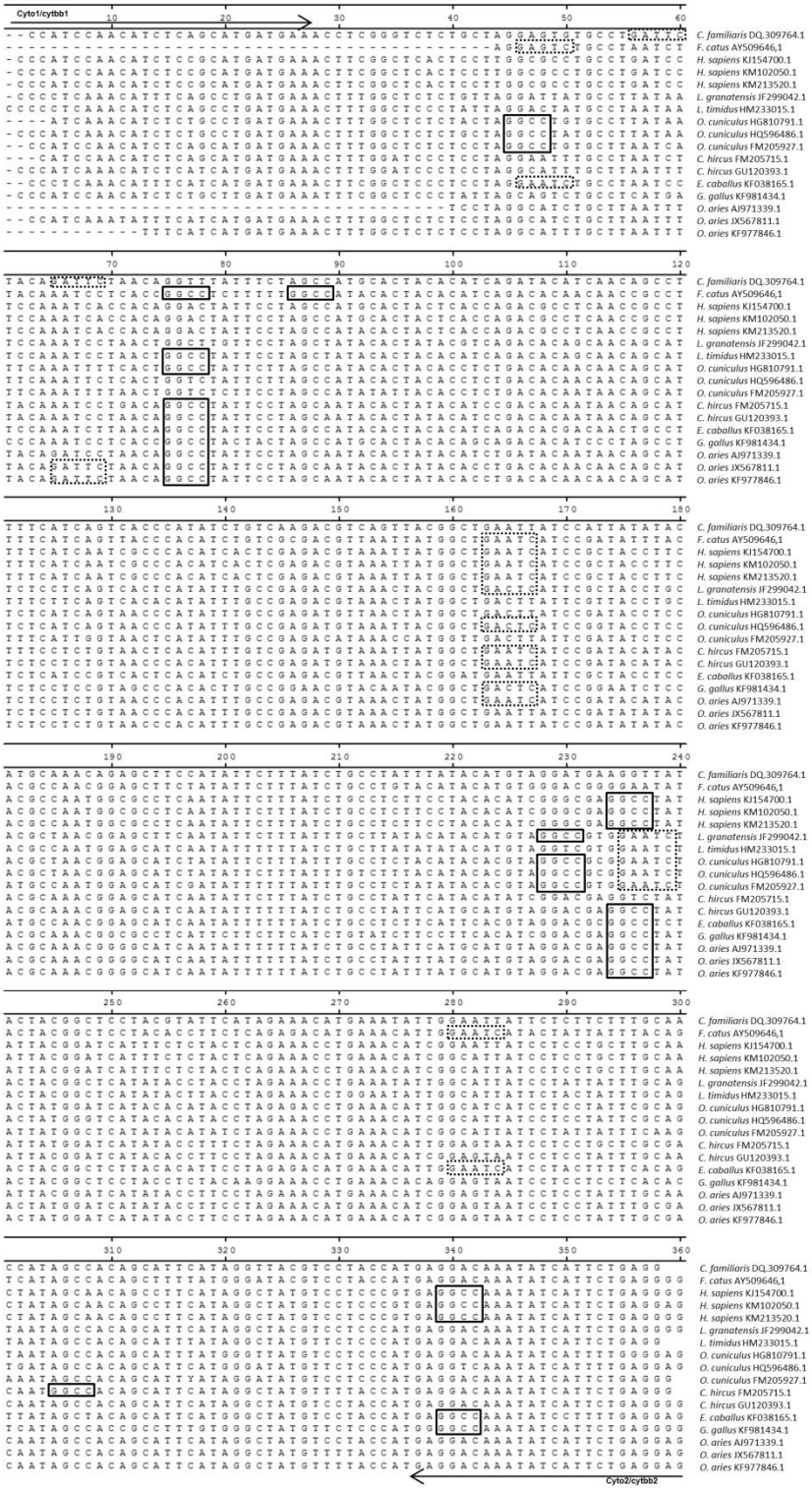

Fig. 1. Alignment of 359 bp*cyt b* fragment of the different species of vertebrates, remarking restriction sites of *Hae*III ('GGCC') (continuous line) and *Hinf*I ('GANTC') (broken line). The following sequences were used: *C. familiaris* (DQ309764.1); *F. catus* (AY509646.1); *H. sapiens* (KJ154700.1, KM102050.1, KM213520.1); *L. granatensis* (JF299042.1) and *L. timidus* (HM233015.1); *O. cuniculus* (HG810791.1, HQ596486.1, FM205927.1); *C. hircus* (FM205715.1, GU120393.1) *E. caballus*

(KF038165.1); *G. gallus* (KF981434.1) and *O. aries* (AJ971339.1, JX567811.1, KF977846.1).

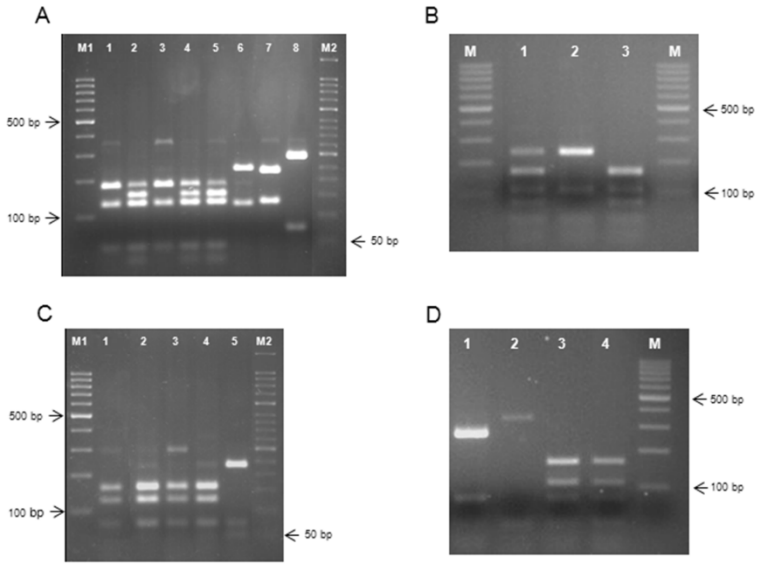

Fig. 2.*Cytb* PCR products digested with restriction enzyme *Hae*III.

A:*Lane M1,* 100 bp molecular weight marker (Biotools), *lane 1 O. cuniculus*(HQ596486.1; 45, 131, 183 bp), *lanes 2, 4 and 5 O. cuniculus*(HG810791.1; 30, 45, 131, 153 bp), *lane 3 O. cuniculus*(FM205927.1; 45, 131, 183 bp), *lane 6 H. sapiens* (20, 105, 234 bp), *lane 7 L. granatensis*(131, 228 bp), *lane 8 L. timidus*(75, 284 bp), *lane M2* 50 bp molecular weight marker (Takara).B:*Lane M* 100 bp molecular weight marker (Biotools), *lane 1 O. aries/H. sapiens, lane 2 H. sapiens*, *lane 2 O. aries*.C:*Lane M1,* 100 bp molecular weight marker (Biotools), lane 1, 2 and 3 mixed blood meal *O. aries/C. hircus, lane 4 C. hircus*(54, 75, 230), *lane 5 O. aries*(75, 125, 159 bp), *lane M2* 50 bp molecular weight marker (Takara).D:*Lane* 1 *F. catus*(11, 20, 74, 254 bp), *lane 2 C.*

familiaris(359 bp), *lanes 3 E. caballus*(20, 75, 105, 159 bp), *lane 4 G. gallus*(20, 75, 105, 159 bp), *lane M* 100 bp molecular weight marker (Biotools).

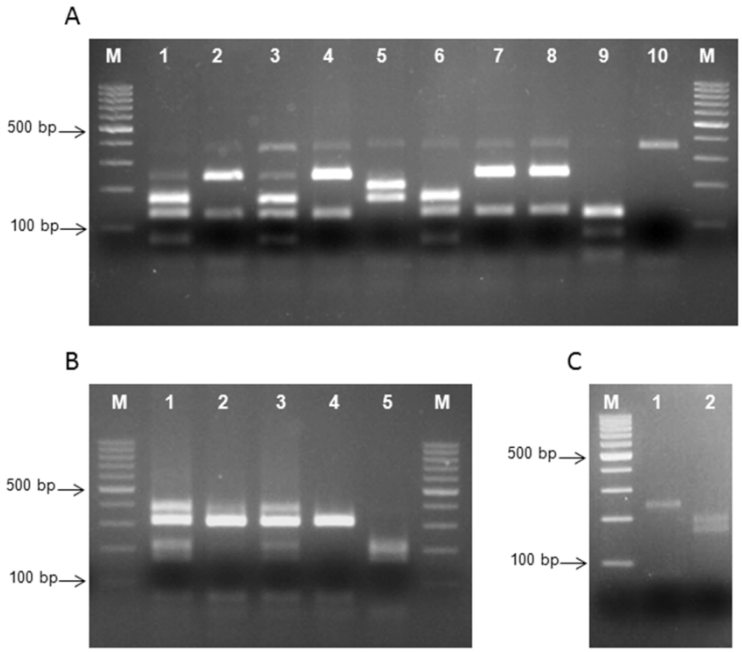

Fig. 3.*Cytb* PCR products digested with restriction enzyme *Hinf*I.

A: *Lane M* 100 bp molecular weight marker (Biotools), *lane 1 O. cuniculus*(HQ596486.1; 72, 125, 162 bp), *lanes 2, 4 and 7 O. cuniculus*(HG810791.1; 124, 235 bp), *lane 3 O. cuniculus*(FM205927.1; 124, 235 bp)*, lane 5 H. sapiens* (162, 197 bp), *lane 6 L. granatensis*(72, 135, 162 bp), *lane 8 L. timidus*(124, 235 bp), *lane 9 F. catus*(45, 79, 117, 117 bp), *lane 10 C. familiaris*(9, 55, 295 bp).B:*Lane M* 100 bp molecular weight marker (Biotools), *lane 1 to 3* mixed blood meal *O. aries/C. hircus, lane 4O. aries*(64, 295 bp), *lane 5 C. hircus*(163, 196

bp).C:*Lane M* 100bp molecular weight marker (Biotools), *lane 1E. caballus*(45, 80, 234 bp), *lane 2 G.gallus*(10, 162, 187 bp).