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Intraspecific variability (rDNA ITS and mtDNA Cyt *b*) of *Phlebotomus sergenti* in Spain and Morocco

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ABSTRACT

Phlebotomus sergenti, the main vector of *Leishmania tropica*, is widely represented in Spain, whilst *L. tropica* is not an endemic species in this country. Nevertheless, the important human migrant flow from regions where *L. tropica* is endemic, the existence of its vector and the anthroponotic cycle of the parasite could lead to its establishment in our country. The vectorial capacity of *P. sergenti* could depend on the existence of cryptic species or races, which can only be identified by molecular methods. Our aim has been to study the molecular variation on two genes of Spanish populations of *P. sergenti*, comparing them with Moroccan ones. We have identified 5 ribosomal and 16 mitochondrial haplotypes. The results obtained indicate a high diversity of *P. sergenti* in Spain and the existence in the country of two *P. sergenti* lineages, a typically Spanish mitochondrial lineage and another one common in Morocco, where *L. tropica* is endemic in the south and emergent in the north and centre of the country.

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1. Introduction

Phlebotomus (*Paraphlebotomus*) *sergenti* Parrot, 1917 is the main vector of *Leishmania tropica* (Wright, 1903), responsible for the anthroponotic cutaneous leishmaniasis (ACL) (Al-Zahrani et al., 1988; Guilvard et al., 1991). It has an extensive geographical distribution, wider than that of the flagellate (Depaquit et al., 2002). It has been suggested that the presence of this sandfly in *L. tropica* free areas and the differences in the transmission patterns of the ACL could be related to the existence of races or even cryptic vector species (Depaquit et al., 2002; Yahia et al., 2004). *Phlebotomus sergenti* is a species widely present in Spain (Gil Collado et al., 1989). In Spain it shows a biphasic phenology in localities with optimal climatology for its development, with two maxima located at the beginning and at the end of the summer period, that are separated by a minimum in the month of August (Martínez Ortega and Conesa Gallego, 1987; Sanchís-Marín et al., 1986), similar to the phenology of this species in Morocco (Guernaoui et al., 2005; Guessous-Idrissi et al., 1997). Conversely, *L. tropica* is not an endemic species in Spain, where only *Leishmania infantum* has this trait (Gállego et al., 2002; Martín-Sánchez et al., 2004). Nevertheless (i) the important human migratory flow from areas where this parasitosis is endemic, (ii) the existence of the vector and (iii) the anthroponotic cycle of the parasite, could lead to its establishment in Spain, in a similar way to what has happened in the north of Morocco (Guessous-Idrissi et al., 1997; Rhajaoui et al., 2004). Bearing in mind that the vectorial capacity of *P. sergenti* could depend on the existence of races or even sibling species, which can be identified only by molecular methods, our aim has been to study the molecular variation of Spanish *P. sergenti* populations, comparing them with Moroccan ones. For that reason, we have selected two genes with a high mutation rate and a different type of heredity, such as are the internal transcribed spacer 2 (ITS2) of the ribosomal DNA (rDNA) and a fragment of the gene Cytochrome *b* (Cyt *b*) of the mitochondrial DNA (mtDNA). In both cases the scientific literature describes primers and conditions for the PCR and with pre-existing knowledge of them in some sandfly species. The ITS2 has provided resolution in several studies at the subgeneric taxonomic level for the *Larroussius* and *Paraphlebotomus* (Depaquit et al., 2000, 2002; Di Muccio et al., 2000). The comparative sequence analysis of mtDNA fragments has been shown to be useful for genetic differentiation of geographical lineages and species on the subgenus *Larroussius*, *Phlebotomus* and *Paraphlebotomus* (Esseghir et al., 1997; Moin-Vaziri et al., 2007; Perrotey et al., 2005; Pesson et al., 2004; Yahia et al., 2004).

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2. Material and methods

2.1. Study areas

The geographical locations from which *P. sergenti* was sampled are shown in Fig. 1. Latitude and longitude, of the different sampled localities, are indicated.

2.2. Collection and morphological identification of specimens

Sandflies were captured overnight with miniature CDC light traps and preserved in liquid nitrogen or at −20 °C until morphological and molecular identification (Table 1). They were slide-mounted in Berlese fluid following dissection and morphologically identified based on external and internal characters of the head and genitalia according to the keys of Gil Collado et al. (1989) and Gállego et al. (1992).

2.3. Sandfly DNA extraction

Genomic DNA was extracted from the thorax and the attached anterior abdomen of individual sandflies (Martín-Sánchez et al., 2000). Briefly, each sandfly was placed in a 1.5-ml Eppendorf tube and kept in liquid nitrogen for a few seconds to facilitate the rupture of cell membranes. The tissue was crushed by a pestle in lysis buffer (10 μl of SCE (sorbitol 1 M, sodium citrate 0.1 M, EDTA 0.06 M), 30 μl of SDS-EDTA (SDS 1%, EDTA 0.15 M) and 10 μl of proteinase K 1%) and then incubated at 42 °C overnight. Subsequently, the DNA was purified with one phenol–chloroform–isoamyl alcohol extraction, followed by one chloroform–isoamyl alcohol extraction and then an ethanol precipitation. The precipitated pellet was resuspended in 50 μl of bidistilled water and kept at −20 °C until use.

2.4. PCR amplification

The polymerase chain reaction was used to amplify a fragment of 418–433 bp containing the ITS2 of sandfly rDNA following the methodology described by Depaquit et al. (2002).

PCR was also used to amplify a 550-bp fragment containing the 3′ end of the mitochondrial gene Cyt *b* following the methodology described by Esseghir et al. (1997). In the specimens in which this PCR did not work, an internal fragment of 442 bp was amplified following the methodology described by Yahia et al. (2004).

2.5. Sequencing and comparative sequence analysis

PCR products were directly sequenced in both directions using the primers used for DNA amplification. Sequences were edited and aligned to identify haplotypes (=unique sequences) using Clustal-X 1.81 software and manually adjusted, if necessary. Poly(AT) was excluded from ITS sequences. Phylogenetic analysis was performed using PHYLIP version 3.65 (http://evolution.genetics.washington.edu/phylip). *Phlebotomus longicuspis* was used as an outgroup.

3. Results

Comparative ITS2 sequence analysis of *P. sergenti* (Table 2): we have analysed this rDNA sequence in 25 *P. sergenti* individuals

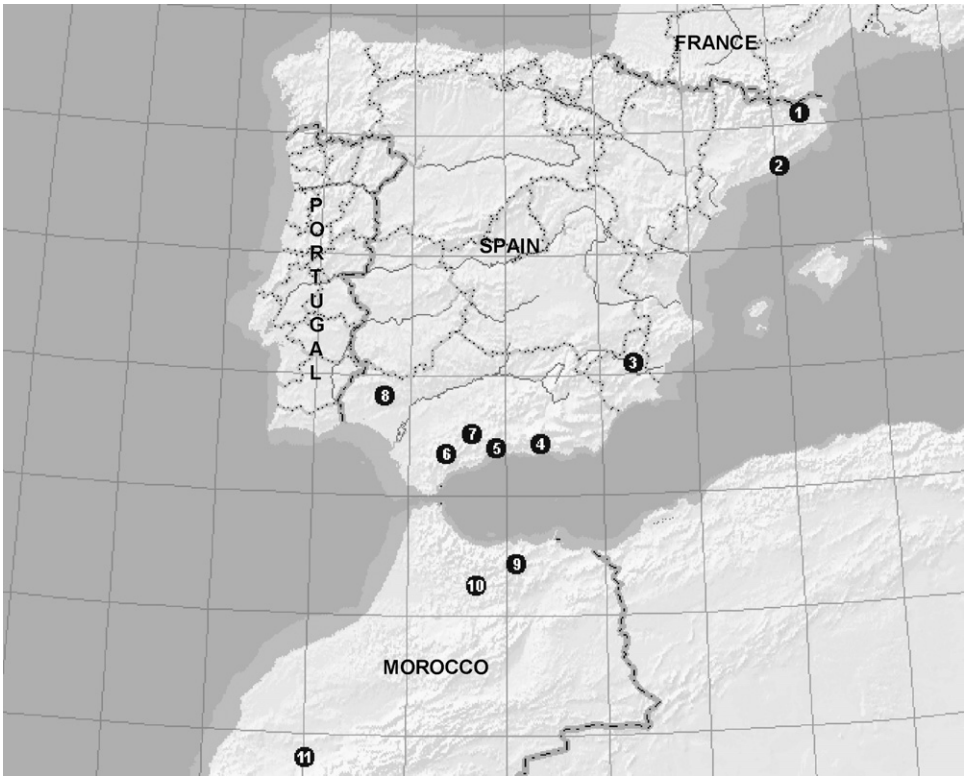

Fig. 1. Geographical locations of the *P. sergenti* sampled populations with indication of the co-ordinates: (1) Sant Jaume de Llierca, Girona province (42°12′N, 2°37′E), (2) Sant Just Desvern, Barcelona province (41°22′N, 2°04′E), (3) Puerto de la Cadena, Murcia province (37°09′N, 1°13′W), (4) Torvizcón, Granada province (36°53′N, 3°18′ W), (5) Almáchar, Málaga province (36°48′N, 4°13′W), (6) Benaoján, Málaga province (36°43′N, 5°15′W), (7) Las Caballerizas, Málaga province (37°02′N, 4°43′W), (8) Riotinto, Huelva province (37°41′N, 6°33′W), (9) Chechaouene, Chaouen province (34°53′ N, 3°49′W), (10) Taounate, Taounate province (34°32′N, 7°59′W) and (11) Dar Bachir, Marrakech province (31°31′N, 7°28′W).

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