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Multilocus molecular and phylogenetic analysis of phlebotomine sand flies (Diptera: Psychodidae) from southern Italy

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ABSTRACT

This study reports a combined analysis of mitochondrial and ribosomal DNA target regions of phlebotomine sand flies (Diptera: Psychodidae) from the Mediterranean region. A ~900 bp long fragment of the mitochondrial DNA encompassing regions within *cytb* and *nd*1 gene and the complete ITS2 ribosomal region (~500 bp) were sequenced and characterized for *Phlebotomus perniciosus*, *Phlebotomus perfiliewi*, *Phlebotomus neglectus*, *Phlebotomus papatasi*, and *Sergentomyia minuta*, captured in two sites of southern Italy. From one to eight mitochondrial haplotypes and from one to three ITS2 sequence types were found for the examined specimens according to the different sand fly species. The mean interspecific difference in the mitochondrial sequences was of 16.1%, with an overall intraspecific nucleotide variation from 0.1 to 2.8%. A higher interspecific difference (mean 25.1%) was recorded for the ITS2 sequence, with an overall intraspecific nucleotide variation up to 4.9%. The sequence types alignment of ITS2 region showed that all phlebotomine specimens possessed a split 5.8S rRNA, consisting of a mature 5.8S rRNA and a 2S rRNA separated by a short transcribed spacer. Phylogenetic analysis of the *Phlebotomus* spp. sequences, herein determined and of those available in GenBank™ were concordant in clustering *P. neglectus*, *P. perfiliewi* and *P. papatasi* with the same species collected from different geographic areas of the Mediterranean basin in four main clades for mtDNA and ITS2, respectively. This study demonstrates the utility of multilocus sequencing, provides a dataset for the molecular identification of the most prevalent phlebotomine sand flies in southern Europe and defines the phylogenetic relationships among species examined.

1. Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are small-sized blood-sucking insects feeding on a wide range of hosts, and potentially acting as vectors of pathogens responsible for human and animal diseases worldwide. Out of over 800 sand fly species that have been described to date, approximately 10% are proven or suspected vectors of bacteria (e.g., *Bartonella bacilliformis*), viruses (e.g., *Phlebovirus*, *Vesiculovirus*) as well as *Leishmania* spp. protozoa (Killick-Kendrick, 1990; Comer and Tesh, 1991; Birtles, 2001; Depaquit et al., 2010). The most prevalent phlebotomine species in the Mediterranean region are *Phlebotomus perniciosus* Newstead, 1911, *Phlebotomus perfiliewi* Parrot, 1930, *Phlebotomus ariasi* Tonnoir, 1921, *Phlebotomus neglectus* Tonnoir, 1921, *Phlebotomus papatasi* (Scopoli, 1786), *Phlebotomus mascittii* Grassi, 1908, *Phlebotomus sergenti* Parrot, 1917, *Phlebotomus longicuspis* Nitzulescu, 1930, *Phlebotomus tobbi* Adler and Theodor, 1930 and *Sergentomyia minuta* (Rondani, 1843) (Killick-Kendrick and Killick-Kendrick, 1999; Maroli et al., 1994; D'Urso et al., 2004). All above listed species but *S. minuta*, are potential vectors of human pathogens. For instance, *P. papatasi* is a vector of *Leishmania major*, the causative agent of the zoonotic cutaneous leishmaniasis in North Africa and Middle East (Lane, 1993), and it has been implicated in the transmission of viruses (e.g., *Phlebovirus*) in Europe (Depaquit et al., 2010). Additionally, *P. ariasi*, *P. longicuspis*, *P. perfiliewi*, *P. perniciosus* and *P. neglectus* have been regarded as proven or suspected vectors of *Leishmania infantum*, which is the causative agent of canine visceral leishmaniasis in the Mediterranean region, and in central-western Asia (Maroli et al., 1987, 1994; Léger et al., 1988, 2000; Doha and Saeta, 1992; Martín-Sánchez et al., 1994; Bongiorno et al., 2003). The occurrence of sand flies in a given region may represent a risk for *L. infantum* transmission or even for the introduction of non-endemic parasite species (e.g., *L. major*) from overseas into the Mediterranean basin (Dujardin et al., 2008), provided that the reservoir host for such species is present in the area.

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The identification of phlebotomine sand flies at species level relies on morphology of key structures including pharynx, spermathecae and cibarium for females and genitalia for males (Killick-Kendrick et al., 1991; Romi et al., 1994). Nonetheless, the morphological identification requires taxonomic expertise and it is time-consuming (Kato et al., 2007). Therefore, molecular techniques, such as random amplified polymorphic DNA (Mukhopadhyay et al., 2000) and polymerase chain reaction-restriction fragment length polymorphism (Barroso et al., 2007) have been used in recent years to differentiate some species of sand flies. In addition, phylogenetic studies have investigated the genetic make-up of single sand fly species (e.g., *P. papatasi*) using ribosomal – 18S (rRNA) and internal transcribed spacer 2 (ITS2) – or mitochondrial – *cytochrome b* (*cytb*) – markers (Aransay et al., 2000; Di Muccio et al., 2000; Depaquit et al., 2000, 2008; Pesson et al., 2004; Hamarsheh et al., 2007). Despite their potential utility in providing an unequivocal identification of sand flies and ulterior information on their ecology and vector capacity, no molecular datasets for mitochondrial and ribosomal DNA gene sequences have been generated for the simultaneous comparison of the most representative phlebotomine sand fly species of the Mediterranean region. Therefore, the objective of the present study was to assess the genetic diversity within and among phlebotomine sand fly species collected in southern Italy and to compare them with those available from Mediterranean region, together with the analysis of their phylogenetic relationships. With this purpose, a long fragment (~900 bp) of the mitochondrial DNA encompassing from *cytb* to *nd*1 and the complete ITS2 ribosomal region were sequenced and characterized for the five sand fly species frequently found in Mediterranean countries.

2. Materials and methods

2.1. Insect source

Sand fly specimens were collected during a previous study (Tarallo et al., 2010) in Putignano municipality (40°51′N, 17°07′E; province of Bari, Apulia region, Italy), while *P. perfiliewi* from the natural park of "Gallipoli Cognato e delle Piccole Dolomiti Lucane" (40°29′N, 16°09′E; Matera province, Basilicata region, Italy). Most of the specimens were collected using sticky traps (Tarallo et al., 2010) whereas *P. perfiliewi* specimens were collected using light traps. Sand flies caught by sticky traps were subjected to serial washing in 90% and 70% ethanol to remove oil before morphological identification. The external genitalia of males and head and posterior end of abdomen (last two tergites) of females were dissected using entomological needles and morphologically identified (Killick-Kendrick et al., 1991; Romi et al., 1994). Voucher slides of each examined specimen are preserved in the parasitological collection at the Faculty of Veterinary Medicine, University of Bari (Italy). The remaining parts of dissected insects were stored in PBS at −20 °C for genomic DNA extraction (Table 1).

2.2. PCR amplification and sequencing

The genomic DNA was extracted from individual females and males by using DNeasy Blood & Tissue Kit (Qiagen, GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. The mitochondrial DNA fragment from *cytb* to *nd*1 regions (~900 bp) was amplified using two primer sets for overlapping regions of about 500 bp each.

Primers used to amplify *cytb* gene are those described in literature, i.e. CB1 (5′-TAT GTA CTA CCA TGA GGA CAA ATA TC-3′) and CB3-RA (5′-GCT ATT ACT CC(T/C) CCT AAC TT(A/G) TT-3′) (Simon et al., 1994; Esseghir et al., 1997). A second fragment encompassing a region from terminal *cytb* to partial *nd*1, was amplified with PhleF (5′-AAT AAA TTA GGA GGA GTA ATT GC-3′) and PhleR (5′-GCC TCG AWT TCG WTT ATG ATA AAT T-3′) primers, which were herein designed, using the criteria of Sharrocks (1994), on the basis of the consensus sequence obtained by the multiple alignment of sequences available in GenBank™. The ITS2 rDNA region (~500 bp) plus part of the 5.8S and 28S flanking regions were amplified using primers JTS3 (5′-CGC AGC TAA CTG TGT GAA ATC-3′) and C1a (5′-CCT GGT TAG TTT CTT TTC CTC CGC T-3′) (Depaquit et al., 2000).

Each reaction consisted of 4 μl genomic DNA and 46 μl of PCR mix containing 2.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.3 and 50 mM KCl, 250 μM of each dNTP, 50 pmol of each primer and 1.25 U of Ampli Taq Gold (Applied Biosystems) using a thermal cycler (2700, Applied Biosystems). Approximately 100 ng of genomic DNA were added to each PCR and samples without DNA were included with each batch of sample tested.The mtDNA fragments were amplified using the following conditions: 94 °C for 12 min (first polymerase activation and denaturation), followed by 5 cycles of 94 °C for 30 s (denaturation); 40 °C for 30 s and 52 °C for 1 min (annealing) for the first and second fragment, respectively, 72 °C for 1 min (extension). A second step PCR included 30 cycles with the same conditions but with 44 °C for 30 s and 54 °C for 1 min (annealing) for the first and second fragment respectively, and a final extension at 72 °C for 10 min. For ITS2, the PCR was carried out at 94 °C for 12 min, followed by 35 cycles of 94 °C for 1 min (denaturation), 62–64 °C (annealing for *P. perniciosus*, *S. minuta*, or for *P. neglectus*, *P. perfiliewi*, *P. papatasi*, respectively) for 1 min and 72 °C for 1 min (extension) followed by a final extension at 72 °C for 10 min. In order to better define the ribosomal sequences, ITS2 of *P. perniciosus* and *S. minuta* were also cloned from PCR products by purifying amplicons of each sample (see below) with a commercial kit (5PRIME, Inc., Gaithersburg, USA). Amplicons were cloned into pGME®-T Easy Vector using the pGME®-T Easy Vector System I (Promega Corporation, Madison, USA). For transformation, JM109 competent *Escherichia coli* cells were used. Recombinant clones containing an insert of the correct size were selected and confirmed by PCR using T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) and SP6 (5′-ATT TAG GTG ACA CTA TAG AA-3′) primers and colonies as template. DNA of the recombinant clones (i.e., 20 for each sample) was extracted using Plasmid Miniprep kit (5PRIME, Inc., Gaithersburg, USA). Amplicons were resolved in ethidium bromide-stained (2%) agarose (Gellyphor, Euroclone, Italy) gels and sized by comparison with markers in the Gene Ruler™ 100 bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania). Gels were photographed by a digital documentation system (Gel Doc 2000, BioRad, UK). Amplicons were purified using Ultrafree-DA columns (Millipore; Bedford, USA) and then sequenced directly using the *Taq* DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems) in an automated sequencer (ABI-PRISM 377).

2.3. Sequence and phylogenetic analyses

Sequences were determined from both strands (using the same primers individually as for the PCR) and the electropherograms verified by eye. In order to ensure open reading frames, all nucleotide sequences of the fragment ranging from partial *cytb* to *nd*1 were conceptually translated into amino acid sequences using the invertebrate mitochondrial code MEGA4 (Tamura et al., 2007). Sequences were aligned using ClustalX program (Thompson et al., 1997). Pairwise comparisons of sequence differences (D) were made using the formula $D = 1 - (M/L)$, where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton et al., 1995).

In order to investigate the relationships between phlebotomine sand flies sequences of both genes with those available GenBank™