Polymerase chain reaction-based assay for the detection and identification of sand fly gregarines in *Lutzomyia longipalpis*, a vector of visceral leishmaniasis

Lorena G. Caligiuri[1,2], Soraya A. Acardi[3], María Soledad Santini[4], Oscar D. Salomón[5], and Christina B. McCarthy[1,2] ✉

[1]*Centro Regional de Estudios Genómicos, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Florencio Varela, Argentina, mccarthychristina@gmail.com*

[2]*Departamento de Informática y Tecnología, Universidad Nacional del Noroeste de la Provincia de Buenos Aires, Pergamino, Buenos Aires, Argentina*

[3]*Laboratorio de Biología Molecular Aplicada, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Posadas, Misiones, Argentina*

[4]*Centro Nacional de Diagnóstico e Investigación en Endemoepidemias, Administración Nacional de Laboratorios e Institutos de Salud, Ministerio de Salud, Buenos Aires, Argentina*

[5]*Instituto Nacional de Medicina Tropical, Ministerio de Salud de la Nación, Puerto Iguazú, Misiones, Argentina*

Received 5 September 2013; Accepted 15 October 2013

ABSTRACT**:** Gregarines that parasitise phlebotomine sand flies belong to the genus *Psychodiella* and, even though they are highly host-specific, only five species have been described to date. Their most outstanding features include the unique localisation of the oocysts in the accessory glands of the female host, which ensures contamination of the egg surface during oviposition, and the fact that they naturally parasitise the vectors of *Leishmania*, causal agent of leishmaniasis. The type species, *Ps. chagasi*, was first described in *Lutzomyia longipalpis*, vector of visceral leishmaniasis (VL), from Brazil. We recently reported *Ps. chagasi* sequences in *Lu. longipalpis* from Posadas (Misiones, Argentina), an endemic VL location where this gregarine had not been previously recorded. In order to analyse the incidence of *Ps. chagasi* infections in *Lu. longipalpis* from this location, the aim of this study was to develop a diagnostic assay for sand fly gregarine parasites in *Lu. longipalpis*. For this, we designed primers using the *Ps. chagasi* sequences we previously identified and performed an *in vitro* validation by PCR amplification of the original sand fly samples. Their specificity and sensitivity as diagnostic primers were subsequently confirmed by PCR reactions using total DNA extracted from naturally infected *Lu. longipalpis* from the same location (Posadas, Argentina). ***Journal of Vector Ecology* 39 (1): 83-93. 2014.**

*Keyword Index: S*and fly gregarines; *Psychodiella chagasi*; diagnostic primers; PCR-based assay; *Lutzomyia longipalpis*; visceral leishmaniasis (VL)

INTRODUCTION

Apicomplexans are very diverse unicellular eukaryotes that parasitize the body cavities and tissues of metazoans. Some apicomplexans, including haemosporidians (e.g., *Plasmodium*), coccidians (e.g., *Toxoplasma* and *Eimeria*) and piroplasms (e.g., *Babesia*), are economically important and a priority in medical research because they are pathogenic for humans and domesticated vertebrates. Gregarines are a distinct group of apicomplexans that are widely distributed in marine and terrestrial invertebrates and are considered to be basal within the apicomplexans (Carreno et al. 1999, Perkins et al. 2000). Their phylogenetic relationships within the Apicomplexa have mostly been determined by comparison of small subunit (SSU) rRNA genes (Carreno et al. 1999, Leander et al. 2003, Votypka et al. 2009, Lantova et al. 2010). Although gregarines currently do not have a recognized impact on human welfare, some species could potentially be used for the biological control of insects (Levine, 1985). This is the case with gregarines that parasitise phlebotomine sand flies, the natural vectors of parasitic protozoa of the genus *Leishmania*, which have been reported in over 20 species of sand flies (Young and Lewis 1977). Only five *Psychodiella* species have been described to date: two New World species, *Ps. chagasi* (Adler and Mayrink 1961) and *Ps. saraviae* (Ostrovska et al. 1990), and three Old World species, *Ps. mackiei* (Shortt and Swaminath 1927), *Ps. sergenti,* and *Ps. tobbi* (Lantova et al. 2010).

The type species of this genus, *Ps. chagasi*, was first described in the hemocele and accessory glands of *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae) from Brazil (Adler and Mayrink 1961) and its life cycle has been studied in detail (Adler and Mayrink 1961, Coelho and Falcao 1964, Wu and Tesh 1989, Warburg and Ostrovska 1991). *Ps. chagasi* lacks intracellular development and completes its life cycle in the larval gut and/or the adult haemocoel. All life stages, including gametocysts and oocysts, occur in larvae and adults of both sexes. During oviposition, the insect egg surface is contaminated with oocysts that are discharged along with secretions from the infected accessory glands; oocysts are glued to the surface of the eggs by the secretion of the glands. Sand fly larvae emerging from contaminated eggs become infected by ingestion of oocysts attached to the egg (Adler and Mayrink 1961) and/or those released into larval habitats after the death and decay of infected adults. Sporozoites, which are released from the oocysts after these are ingested by sand fly larvae, attach to the gut epithelial cells and develop into trophozoites. In adults, gregarines

which are located in the body cavity undergo sexual development. In females, gametocysts attach to the accessory glands and oocysts are injected into their lumen. This general life cycle is modified in other *Psychodiella* species and differences have been described in *Ps. mackiei* (Shortt and Swaminath 1927) and *Ps. sergenti* (Lantova et al. 2010, Lantova and Volf 2012).

Ps. chagasi is particularly interesting because it is a parasite of *Lu. longipalpis* (Adler and Mayrink 1961), the main vector in the New World of *Leishmania infantum* (syn. *chagasi*), which is the causal agent of visceral leishmaniasis (VL) (Sharma and Singh 2008). Leishmaniasis is a vector-borne neglected infectious disease of worldwide incidence with great importance in human and veterinary medicine. VL causes an estimated 500,000 new cases and more than 59,000 deaths each year, a death toll that is only surpassed by malaria among the parasitic diseases. *Lu. longipalpis* is only found in the New World, with a wide distribution from Mexico to Argentina (Grimaldi et al. 1989). We recently used unbiased high-throughput pyrosequencing technology to compare the diversity of taxa associated with wild male and female *Lu. longipalpis* from Posadas (Misiones) and Lapinha Cave (Minas Gerais), in Argentina and Brazil, respectively (McCarty et al. 2011). In this study we identified sequences from bacteria, fungi, protists, plants, and metazoans. In particular, *Ps. chagasi* was found in male *Lu. longipalpis* from Posadas. Considering biocontrol strategies seek to reduce target population sizes to levels in which they are no longer a health problem (Service 1985), various features make *Ps. chagasi* an interesting candidate for the biological control of *Lu. longipalpis*. In the first place, as mentioned previously, it has two life cycle modes which constitute an efficient mechanism for its perpetuation (Adler and Mayrink 1961). Infections in larvae contribute to horizontal as well as vertical transmission to non-offspring (larva to larva), whereas infections in adults facilitate the geographic dispersal of the parasite and ensure an efficient vertical transmission (adult to larva) due to the contamination of eggs during oviposition. In the second place, *Ps. chagasi* has a very narrow host range (Adler and Mayrink 1961, Wu and Tesh 1989), which is another desirable feature in a biocontrol agent. In the third place, it has been found to reduce *Lu. longipalpis* survival (Wu and Tesh 1989) and, under intensive rearing conditions in the laboratory, it reduces longevity and egg production and is thought to be a major contributing factor to the phenomenon of colony crashes (Dougherty and Ward 1991).

Given the fact that we previously found *Ps. chagasi* sequences in wild *Lu. longipalpis* from Posadas (Misiones, Argentina), a location where this parasite had not been reported before, and in order to analyze its incidence, the objective of this study was to develop a simple diagnostic assay for sand fly gregarine parasites in *Lu. longipalpis*. Additionally, even though the original *Ps. chagasi* sequences that we identified corresponded to RNA (McCarty et al. 2011), which suggested that this parasite was biologically active, we wanted to verify that the gregarine was completing its life cycle in these sand flies.

MATERIALS AND METHODS

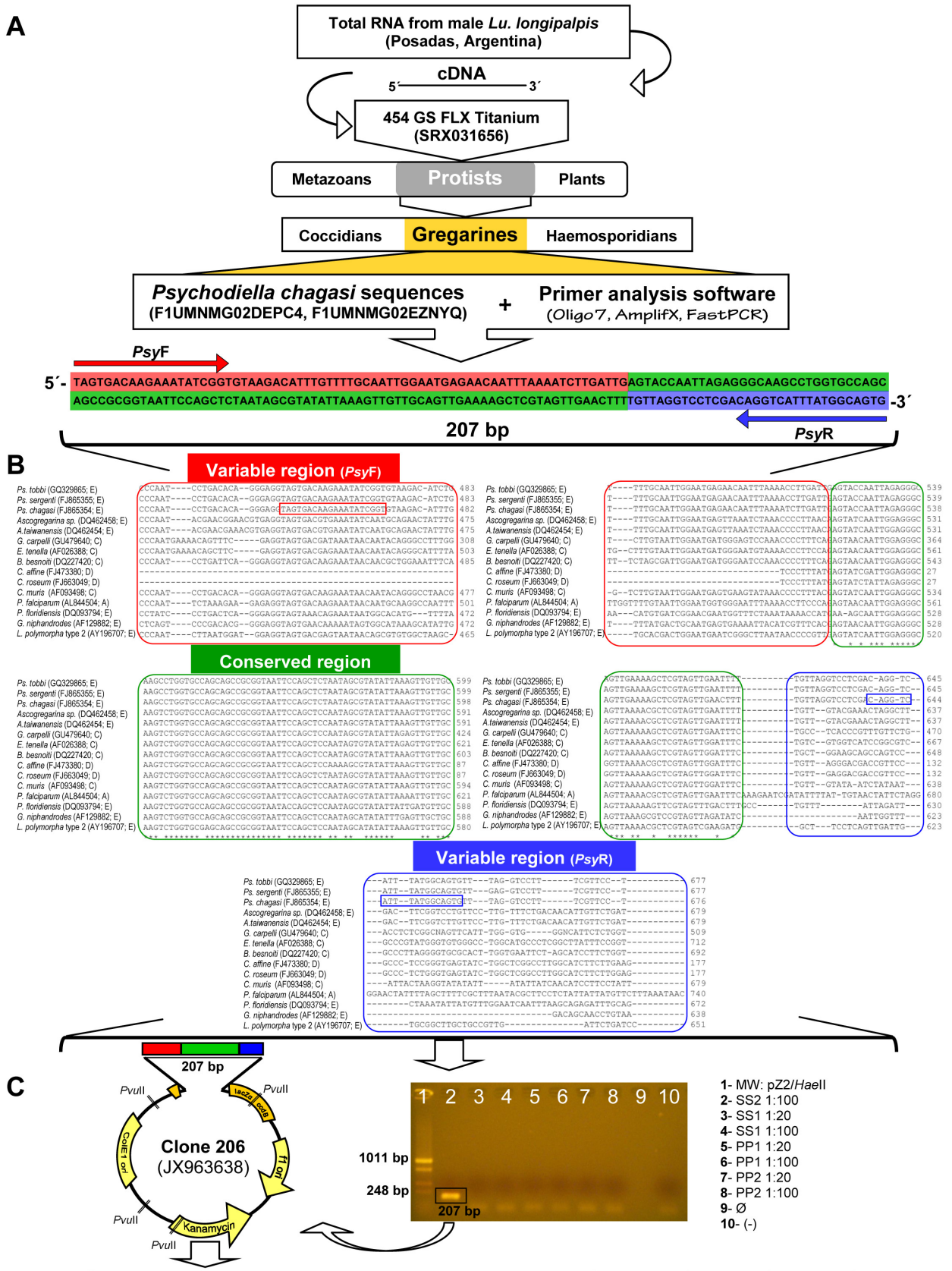
Primer design and *in silico* analysis

The primers were designed based on two of the *Ps. chagasi* SSU rDNA sequences (F1UMNMG02DEPC4 and F1UMNMG02EZNYQ) which we previously identified in total RNA extracted from male *Lu. longipalpis* from Posadas (Misiones, Argentina) (accession SRX031656) (McCarty et al. 2011). Various software programs for primer analysis were used, including Oligo7, FastPCR and AmplifX, and parameters such as annealing temperature, length, amplification product length, and theoretical specificity were taken into account to choose an optimal primer pair (Figure 1). The selected primer pair consisting of *Psy*F (forward; 5′-TAGTGACAAGAAATATCGGT-3′; melting temperature (*T*m) = 50.4° C), and *Psy*R (reverse; 5′-CACTGCCATAAATGACCTG-3′; *T*m = 52.9° C) was designed to amplify a theoretical fragment of 207 base pairs (bp). An *in silico* analysis of these primers was performed on the basis of a multiple sequence alignment including 13 apicomplexan (gregarines, coccidians, and haemosporidians) and two dinoflagellate SSU rDNA full length sequences which were aligned using ClustalW (Thompson et al. 1994) (Figure 1).

In vitro analysis of the designed primers

Amplifications were carried out with the *Psy*F-*Psy*R primer

Figure 1. Overview of the design (A), *in silico* analysis (B), and *in vitro* validation (C) of the diagnostic *Psychodiella* primers.
(A) Primer pair *Psy*F-*Psy*R was designed on the basis of *Ps. chagasi* sequences which we previously identified in total RNA extracted from male *Lu. longipalpis* from Posadas (Argentina) (McCarthy et al., 2011). The (color-coded) theoretical 207 bp sequence targeted by the primers is shown: sequences targeted by primers *Psy*F and *Psy*R are indicated on top of the sequence by red and blue arrows, respectively. Nucleotides highlighted by pale red and pale blue solid rectangles indicate variable regions (see below), and nucleotides highlighted by a pale green solid rectangle indicate the conserved region of the fragment (see below).
B) The *in silico* analysis was based on a multiple sequence alignment (MSA) of 13 apicomplexan and two dinoflagellate SSU rDNA full-length sequences. The schematic only includes the MSA region covering the targeted DNA fragment and adjacent sequences. The targeted DNA sequence comprised a central conserved region (indicated by green boxes in the MSA, which outline the nucleotides involved in this region, as well as a green solid rectangle on the upper part of the MSA) and variable flanking regions (indicated by red and blue boxes in the MSA, which outline the nucleotides involved in these regions, as well as red and blue solid rectangles on the upper part of the MSA). The sequences targeted by primers *Psy*F and *Psy*R are highlighted by red and blue rectangles, respectively, in the *Ps. chagasi* sequence. The following information is provided for each sequence in the MSA: species, accession number, and Class. E: Class Gregarinia, Order Eugregarinida; C: Class Coccidia, Order Eucoccidiorida; A: Class Aconoidasida, Order Haemosporida; D: Class Dinophyceae, Order Coccidiniales.
C) For the *in vitro* validation of primers *Psy*F and *Psy*R, the original cDNA obtained from male and female *Lu. longipalpis* from Posadas and Lapinha Cave (McCarthy et al., 2011) was submitted to PCR amplification. The photograph shows the amplification results visualized on a 1% agarose gel, in which a single DNA band of the expected size (207 bp) was only amplified in males from Posadas (see text for details). The amplified DNA fragment was gel-purified, cloned into pZErO™-2 (Invitrogen) and sequenced. The bottom part of this section shows the best hit which was retrieved after homology searches (Altschul et al., 1990) using the cloned sequence (clone 206; AN JX963638) as query against DB:nt. MW: pZ2/*Hae*II: molecular weight, pZErO™-2 digested with *Hae*II; SS2: cDNA amplified from total RNA extracted from male *Lu. longipalpis* from Posadas (Misiones, Argentina); SS1: cDNA amplified from total RNA extracted from female *Lu. longipalpis* from Posadas (Misiones, Argentina); PP1: cDNA amplified from total RNA extracted from female *Lu. longipalpis* from Lapinha Cave (Minas Gerais, Brazil); PP2: cDNA amplified from total RNA extracted from male *Lu. longipalpis* from Lapinha Cave (Minas Gerais, Brazil); 1:20 and 1:100: dilutions; Ø: empty lane; (-): PCR negative control; DB:nt: NCBI non-redundant nucleotide database.



Sample	Best blastn hit against DB:nt	Coverage	E-value	Identity
Clone 206 (JX963638)	*Psychodiella chagasi* small subunit ribosomal RNA gene, partial sequence (GenBank: FJ865354.1).	92%	2e-63	100%

pair using different dilutions (1:20 and 1:100) of the cDNA previously amplified from total RNA extracted from male and female *Lu. longipalpis* from Posadas (Misiones, Argentina) and Lapinha Cave (Minas Gerais, Brazil) (McCarty et al. 2011) as the template (Figure 1). Amplifications were also carried out using different dilutions of *Plasmodium falciparum* DNA from strains NF54 and 134 as the template.

Amplifications were completed in a ThermoHybaid MBS 0,2S PCR Thermal Cycler. The reaction mixture contained 1X PCR buffer (Tris-HCl 200 mM; pH 8.4; KCl 500mM); either 2 mM or 2.5 mM $MgCl_2$; 0.125 mM dNTPs; 0.03 U/µl Taq Pegasus® DNA polymerase (Productos Bio-Lógicos, Argentina); 0.5 µM of each primer (*Psy*F and *Psy*R); either 0.1 mg/ml bovine serum albumin (BSA) or no BSA, and template DNA, in final volumes of either 10 or 25 µl. The following profile was used: an initial denaturation cycle at 94° C for 5 min, followed by 35 cycles with denaturation at 94° C for 30 s, 52° C for 30 s, and 74° C for 1 min, and a final extension cycle at 74° C for 7 min. PCR products were visualized on a 1% agarose gel (Figure 1) and an amplified fragment of the expected size was gel purified using a siliconized glass fiber method (Vaux 1992). The purified DNA fragment was then cloned into pZErO™-2 (Invitrogen) (Figure 1). Putative clones selected by restriction analysis were sequenced (Macrogen Inc., Korea) and submitted to homology searches (Altschul et al., 1990) against the NCBI non-redundant nucleotide database (DB:nt) (Figure 1).

The cloned *Ps. chagasi* sequence (amplified from cDNA previously obtained from male *Lu. longipalpis* from Posadas (McCarty et al. 2011) and cloned into pZErO™-2 (Invitrogen); clone 206) was deposited in GenBank under accession number (AN) JX963638.

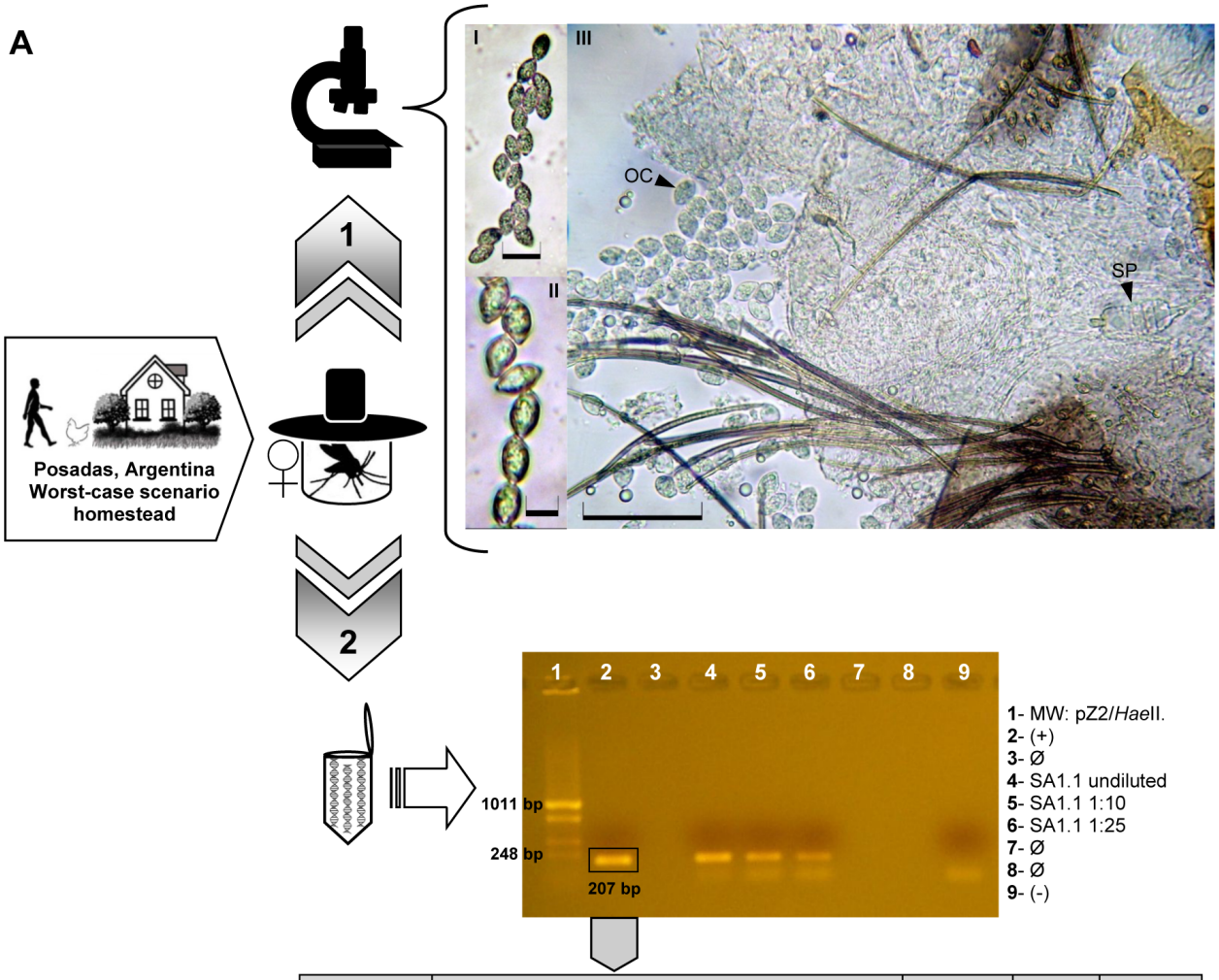
Sand fly field sampling and specimen preparation

Sand flies were captured using mini-CDC light traps (Sudia and Chamberlain 1962) in the city of Posadas (Misiones, Argentina; 27°23'S; 55°53'W, 120 m above sea level), an endemic VL location where *Lu. longipalpis* is distributed in patches of high and low abundance (Fernandez et al. 2010). The traps were located in home peridomiciles that fulfilled the "worst scenario" criteria, i.e., had a peridomestic environment that was most likely to have phlebotomine sand flies (Feliciangeli et al. 2006, Correa Antonialli et al. 2007), and in which owners gave permission for the study to be conducted on their land. The sampling effort was carried out for other studies (Acardi et al. 2010, Fernandez et al. 2010, Salomon et al. 2010, Santini et al. 2010, Santini et al. 2012) during December, 2010, and included 38 sampling sites that were each sampled during two consecutive nights depending on weather conditions. Sites were selected according to the previously described high and low abundance areas (Fernandez et al., 2010). The peridomicile of the only sampling site where infected sand flies were captured (27°22'3.96"S, 55°55'33.78"O) (Table 1) included a poultry house with 18 hens, which had shade all day, orange, lemon, and banana trees that were separated by 2-5 m, and a clean patio earth floor with no leaves or accumulated organic material. Sand flies were transported refrigerated in a nylon cage to the laboratory in Posadas (Misiones) where they were killed at low temperature. Taxonomic identification was performed according to Young and Duncan (1994) with the modification of (Andrade Filho et al. 2003). To avoid cross-contamination, sand flies were determined separately on individual slides. Sand flies were separated according to sex and presence/absence of gregarine infection, and stored at -20º C. Prior to DNA extraction, 70% ethanol was added and the sand flies were stored at -20º C until the extraction process.

Light microscopy analysis

Sand flies that showed gregarine infection during taxonomic identification were further analysed under an optical microscope (JENA, Carl Zeiss) in phosphate buffered saline (PBS) (Figure 2). The shape and size of gregarine oocysts were observed and measured under an optical microscope (JENA, Carl Zeiss). Light micrographs were taken with an FE-100 digital camera (Olympus) (Figure 2) and measurements were processed with Piximetre version 5.4 (http://ach.log.free.fr/Piximetre). Length (L) and width (W) were measured and the length/width ratio (L/W) was calculated for 50 oocysts. Mean and standard deviation (SD) were calculated for each of these parameters. Taxonomic identification of the gregarines was performed by comparison of oocyst

Figure 2. Field validation of the diagnostic PCR-based assay for sand fly gregarines in *Lu. longipalpis*.
A) *Sand fly sampling, total DNA extraction, PCR amplification and sequencing:* Female *Lu. longipalpis* were captured in the peridomicile of a worst-case scenario homestead in Posadas (see text for details). (1) Routine dissection and light microscopy analysis revealed gregarine infection in six of these female sand flies (see text for details). Light micrographs show the *Ps. chagasi* oocysts found in one of the dissected females. Scale bars: 20 µm (I), 10 µm (II) and 50 µm (III). OC: oocysts; SP: spermatheca. (2) Total DNA was subsequently extracted from these females and submitted to PCR amplification with primers *Psy*F and *Psy*R. Amplification products were visualised on a 1% agarose gel, where a single band of the expected size (207 bp) was amplified from the different dilutions. The PCR product was sequenced and submitted to BLASTN (Altschul et al., 1990) analysis. The best hit from the homology searches (Altschul et al., 1990) is shown (see text for details). MW: pZ2/*Hae*II: molecular weight, pZErO™-2 digested with *Hae*II; (+): PCR positive control, clone 206 (JX963638) 1:100 dilution; SA1.1 (undiluted, 1:10 and 1:25 dilutions): total DNA extracted from five infected females from Posadas; Ø: empty lane; (-): PCR negative control.
B) *Molecular Phylogenetic anaylsis by Maximum Likelihood method:* The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). The bootstrap consensus tree inferred from 1,000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one-fourth of the total number of sites, the maximum parsimony method was used; otherwise, the BIONJ method (Gascuel 1997) with a Maximum Composite Likelihood (MCL) distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (four categories (+G, parameter = 0.6975)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 138 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). The sequence reported in this study used to conduct this evolutionary analysis is highlighted in bold (SA1.1). Sand fly and mosquito gregarines are indicated with brackets. The following information is provided for each sequence: species, accession number, Class, and host organism. E: Class Gregarinia, Order Eugregarinida; N: Class Gregarinia, Order Neogregarinida; C: Class Coccidia, Order Eucoccidiorida; D: Class Dinophyceae, Order Coccidiniales; P: Class Dinophyceae, Order Prorocentrales and G: Class Dinophyceae, Order Gymnodiniales. The bar represents 0.05 substitutions per site.



Sample	Best BLASTN hit against DB:nt	Coverage	E-value	Identity
SA1.1 (KC113486)	*Psychodiella chagasi* small subunit ribosomal RNA gene, partial sequence (GenBank: FJ865354.1).	89%	3e-73	98%

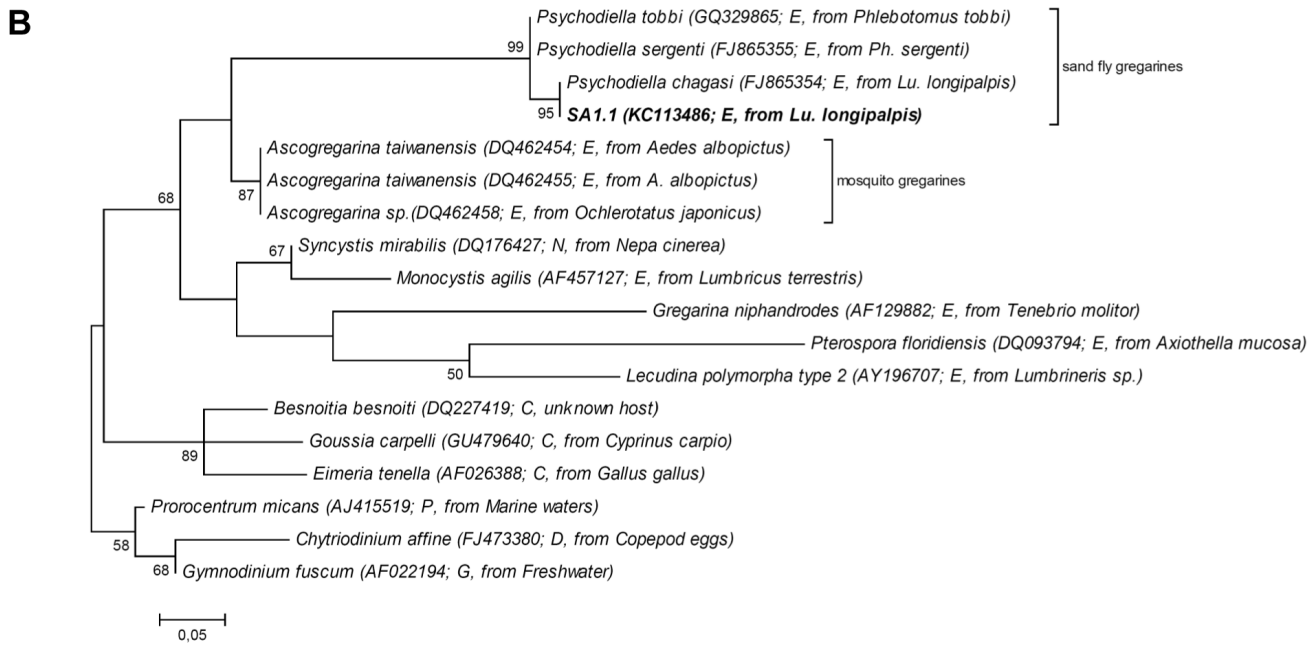


Table 1. Detail of female *Lu. longipalpis* captures in the only sampling site in Posadas where *Ps. chagasi* infections were detected during December, 2010.

Sampling date (dd/mm/yyyy)	Number of captured female *Lu. longipalpis*	Number of females with gregarine infection
02/12/2010	0	None
05/12/2010[a]	48	6

[a]The first day with no rain after the first sampling night on 02/12/2010.

dimensions with those recently reported for *Ps. chagasi*, *Ps. tobbi* and *Ps. sergenti* (Lantova et al. 2010). An independent groups t-test for unequal variance was performed to compare mean L, W, and L/W between oocysts (Table 2).

DNA extraction, PCR amplification, and sequencing

For total DNA extraction, three separate pools of sand flies, two composed of one and five infected females each and the third composed of five females with no apparent infection, were macerated, ground with a Teflon pestle, and DNA was extracted according to de Pita-Pereira et al. (2005) with the modifications of Acardi *et al.* (2010) (Figure 2). DNA pellets were resuspended in 20 µl DNAse/RNAse-free distilled water and stored at -20º C until later analysis. The samples were named SA1.1 (five infected females), SA1.2 (one infected female), and CM1.5 (five uninfected females).

An internal PCR control was implemented to confirm the efficiency and quality of the DNA extractions, using published primers that amplify the IVS6 region of the *Lu. longipalpis* constitutive cacophony gene (Lins et al. 2002) (data not shown). Subsequently, amplifications with primers *Psy*F and *Psy*R were carried out according to the above conditions using the extracted DNA as template (undiluted and various dilutions). PCR products were visualised on a 1% agarose gel and, for those samples in which a DNA fragment was amplified, the rest of the amplification product was sequenced (Macrogen Inc., Korea). Sequences were submitted to BLASTN analysis (Altschul et al., 1990) against DB:nt (Figure 2).

The *Ps. chagasi* sequence amplified from infected female *Lu. longipalpis* from Posadas (Misiones) (SA1.1) was deposited in GenBank under AN KC113486.

Sequence analysis

A Maximum Likelihood (ML) analysis was conducted to verify the phylogenetic position of the DNA sequence amplified from SA1.1 (AN KC113486). For this, a dataset comprised of SA1.1 and reference sequences from GenBank, including 14 apicomplexan sequences (*Ps. tobbi* AN Q329865, *Ps. sergenti* AN FJ865355, *Ps. chagasi* AN FJ865354, *Ascogregarina taiwanensis* AN DQ462454, *A. taiwanensis* AN DQ462455, *Ascogregarina* sp. AN DQ462458, *Syncystis mirabilis* AN DQ176427, *Monocystis agilis* AN AF457127, *Gregarina niphandrodes* AN AF129882, *Pterospora floridiensis* AN DQ093794, *Lecudina polymorpha* type 2 AN AY196707, *Besnoitia besnoiti* AN DQ227419, *Goussia carpelli* AN GU479640, and *Eimeria tenella* AN AF026388) and three dinoflagellate sequences that were used as outgroups (*Prorocentrum micans* AN AJ415519, *Chytriodinium affine* AN FJ473380, and *Gymnodinium fuscum* AN AF022194) (Figure 2), were manually edited using BioEdit version 7.0.5.3 (Hall, 1999) and aligned using ClustalW (Thompson et al. 1994). JModelTest

Table 2. Size comparison between oocysts found in *Lu. longipalpis* from Posadas and *Ps. chagasi* oocysts (Lantova et al. 2010).

Values	Oocysts	Mean	Min.	Max	SD	t	df	p
L/W	Posadas	1.55	1.35	1.76	0.09	2.155	68.99	0.035
	Ps. chagasi (Lantova et al. 2010)	1.52	1.41	1.68	0.06			
L	Posadas	12.57	11.01	13.69	0.59	1.476	60.51	0.145
	Ps. chagasi (Lantova et al. 2010)	12.7	12.0	13.3	0.3			
W	Posadas	8.14	7.30	8.84	0.43	2.387	70.94	0.020
	Ps. chagasi (Lantova et al. 2010)	8.3	7.3	8.9	0.3			

An independent groups t-test for unequal variance was performed to compare mean values (length, width, and length/width ratio) between oocysts found in *Lu. longipalpis* from Posadas and those published for *Ps. chagasi* (Lantova et al., 2010). *N*=50 and *N*=113 are the sample sizes for oocysts from Posadas and *Ps. chagasi* oocysts (Lantova et al., 2010), respectively. Measurements are expressed in µm. L/W: length/width ratio; L: length; W: width; Min.: minimum measured value; Max.: maximum measured value; SD: standard deviation; t: t value; df: degrees of freedom; p: two-tailed probability.

software version 0.1.1 (Posada and Buckley 2004, Posada 2008) was used to select the nucleotide substitution model that best fit the data according to the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). The evolutionary history was inferred by using the Maximum Likelihood method based on the two recommended models, Hasegawa-Kishino-Yano nucleotide substitution model (Hasegawa et al., 1985) (HKY with its variants +G and +I+G) and Kimura-2 parameter model (Kimura, 1980) (K80 with its variants +G and +I+G). Evolutionary analyses were conducted in MEGA5 version 5.05 (Tamura et al., 2011). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein 1985). The analysis involved 18 nucleotide sequences (Figure 2). All positions containing gaps and missing data were eliminated. There were a total of 138 positions in the final dataset.

RESULTS

Design, *in silico* analysis, and *in vitro* validation of diagnostic *Psychodiella* primers

Figure 1 shows a schematic of the steps involved in this first stage of analysis. In order to further analyse gregarine infection in *Lu. longipalpis* from Posadas, we designed diagnostic primers *Psy*F and *Psy*R on the basis of *Ps. chagasi* sequences we previously identified in male *Lu. longipalpis* from Posadas (McCarty et al. 2011) (Figure 1A). An *in silico* analysis of the DNA fragment amplified by these primers revealed that it comprises a central conserved region and variable flanking regions (Figure 1B). Sequences targeted by the designed primers are included in the variable flanking regions and, since *Psy*R only anneals successfully in the case of *Psychodiella* sp., this accounts for the primer pair specificity at the genus level (Figure 1B).

For the *in vitro* validation of the designed primers, PCR amplifications were performed under different reaction conditions using the original cDNA obtained from male and female *Lu. longipalpis* from Posadas and Lapinha Cave (McCarty et al. 2011) as template (Figure 1C). Various taxa were previously identified by sequence homology in these samples (McCarthy et al. 2011): apicomplexans (coccidians, gregarines -*Ascogregarina* and *Psychodiella*-, and haemosporidians), plants and metazoans (human and chicken) in males from Posadas, Apicomplexa (coccidian), bacteria, plants and metazoans (human) in females from Posadas, bacteria, fungi, plants and metazoans (human) in males from Lapinha Cave, and bacteria, fungi, plants, and metazoans (human, chicken, and lizard) in females from Lapinha Cave. Amplification results were consistent in every case: a single DNA band of the expected size was only amplified in males from Posadas (1:20 and 1:100 dilutions) and there was no amplification in the other samples even with the more concentrated 1:20 dilutions (Figure 1C). This result was as expected because *Ps. chagasi* sequences were only identified in males from Posadas (McCarthy et al. 2011). The amplified DNA fragment was purified, cloned, and sequenced, and BLASTN analysis (Altschul et al., 1990) confirmed its homology to the *Ps. chagasi* SSU rRNA gene (Figure 1C). The *in vitro* specificity of *Psy*F and *Psy*R was further confirmed by PCR amplifications using different dilutions of *P. falciparum* DNA from strains NE54 and 134 as a template, which yielded no amplification product (data not shown).

Confirmation of gregarine infection in *Lu. longipalpis* from Posadas (Misiones, Argentina)

A total of 59 female *Lu. longipalpis* was captured in the city of Posadas (Misiones, Argentina) during December, 2010 (Acardi et al. 2010, Fernandez et al. 2010, Salomon et al. 2010, Santini et al. 2010, Santini et al. 2012). Gregarine infection was observed during routine dissections, as was the case for *Ps. chagasi* when it was first described (Adler and Mayrink 1961). Oocysts were found in six females that were captured in one of the sites from a high abundance area patch (Fernandez et al., 2010), where a total of 48 females were caught (see Table 1 for details). Approximately 100 oocysts were observed in each of the infected females. The morphology and dimensions of the oocysts (Figure 2A.1) were compared to those recently published for *Ps. chagasi*, *Ps. tobbi* and *Ps. sergenti* (Lantova et al. 2010). An independent groups t-test for unequal variance was performed to compare the mean values (L, W, and L/W) between the oocysts found in *Lu. longipalpis* from Posadas (Posadas oocysts) and *Ps. chagasi*, *Ps. tobbi,* and *Ps. sergenti* oocysts (Lantova et al. 2010). Posadas oocysts were significantly different from both *Ps. tobbi,* and *Ps. sergenti* oocysts (L, W, and L/W; data not shown), indicating Posadas oocysts were neither *Ps. tobbi* nor *Ps. sergenti* oocysts. The t-statistics for Posadas oocysts/*Ps. chagasi* oocysts was not significant at the 0.01 critical alpha level ($p>0.01$; Table 2), indicating that the oocysts found in these female *Lu. longipalpis* from Posadas were *Ps. chagasi* oocysts (Table 2, Figure 2A.1).

Field validation of the PCR-based assay for detecting *Psychodiella* sp. in *Lu. longipalpis*

To determine the suitability of the PCR-based assay for identifying natural infections of *Psychodiella* sp. in *Lu. longipalpis*, we considered three crucial aspects: the DNA extraction process (to establish if it interfered with amplification and detection), specificity of the primers (when used to amplify total DNA extracted from an environmental source), and limit of detection of the assay (the minimum amount of target DNA sequence detected in a sample). In this context, DNA was extracted from three pools of sand flies using the aforementioned protocol (de Pita-Pereira et al. 2005, Acardi et al. 2010): SA1.2, one female infected with around 100 oocysts; SA1.1, five infected females (approximately 500 oocysts in total); and CM1.5, five females with no apparent infection. To analyze the first two crucial aspects (determining if the DNA extraction process interfered with amplification and detection, and if the primers were specific when used to amplify total DNA extracted from an environmental source), the DNA extractions (undiluted and various dilutions) were used as a template in PCR reactions with primers *Psy*F and *Psy*R under various conditions. Results were consistent in every case: a single DNA fragment of the expected size was amplified in the reactions where DNA extracted from the infected female sand flies was used (SA1.1 and SA1.2; Figure 2.A.2), whereas there was no amplification in the reactions where DNA extracted from the uninfected sand flies was used (CM1.5; data not shown). The PCR amplification product was sequenced and BLASTN analysis (Altschul et al., 1990) showed maximum identity of the DNA sequence with the *Ps. chagasi* SSU rRNA gene (Figure 2.A.2). To determine the third aspect (detection limit of the assay)

and following the rationale applied in previous work (Acardi et al. 2010), serial dilutions of the DNA extracted from the single female infected with *Ps. chagasi* (SA1.2; 10^{-1} to 10^{-4}) were used as a template and the detection limit was estimated at 0.1 femtograms.

Phylogenetic analysis

In order to verify the phylogenetic position of the DNA sequence, we amplified from infected female *Lu. longipalpis* from Posadas (SA1.1) and inferred the evolutionary history using the Maximum Likelihood method. Analyses were carried out using two different nucleotide substitution models (HKY (Hasegawa et al. 1985) and K80 (Kimura 1980) and the same evolutionary history was observed in every case (data are only shown for the HKY+G model; Figure 2B). These analyses revealed that our sequence (SA1.1) formed a well supported clade with *Ps. chagasi* and confirmed its closer relationship to the New World *Ps. chagasi* found in *Lu. longipalpis*, with a high bootstrap value (95%), than to the Old World *Ps. sergenti* and *Ps. tobbi* from *Phlebotomus sergenti* and *Ph. tobbi*, respectively (Figure 2B). Furthermore, the fact that our sequence formed a well supported clade with *Ps. chagasi* from *Lu. longipalpis* indicated that the region targeted by the designed primers (*Psy*F and *Psy*R), of only 207 bp, was appropriate for identifying *Psychodiella* sp.

DISCUSSION

This is the first study to develop a PCR-based assay for the detection and identification of natural gregarine infections in *Lu. longipalpis*. For this, sand fly gregarine diagnostic primers *Psy*F and *Psy*R were designed on the basis of *Ps. chagasi* SSU rDNA sequences we previously identified in male *Lu. longipalpis* from Posadas (McCarthy et al. 2011). The diagnostic primers amplify a 207 bp fragment of the *Psychodiella* SSU rRNA gene (Figure 1.A) and their specificity and sensitivity were confirmed by *in silico* analysis (Figure 1.B) and *in vitro* (Figure 1.C) and field validations (Figure 2).

Ps. chagasi natural infections have previously only been recorded in *Lu. longipalpis* from Lapinha (Minas Gerais) (Adler and Mayrink 1961) and Jacobina (Bahia) in Brazil (Lantova et al. 2010). Given what is currently known about the pathogenicity of gregarines, these parasites are possible biocontrol agents and various strategies have been explored. Different studies in mosquito gregarines have shown that host mortality increases under stressful larval environments (Comiskey et al. 1999a, Comiskey et al. 1999b, Tseng 2004) and high doses (Sulaiman 1992). Similarly, a recent study showed that *Ps. sergenti* is harmful to its host *Ph. sergenti* under laboratory conditions and that the parasite's effects can be influenced by environmental factors (Lantova et al. 2011). Furthermore, the severity of a gregarine infection (*Ascogregarina culicis*) on its natural host (*Aedes aegypti*) was shown to vary both among four different geographic origins of the parasite and among three different populations of *Ae. aegypti* (Sulaiman 1992). Likewise, adult infection rates and intensity of infection varied significantly between two strains of *Lu. longipalpis* from Colombia and Brazil (Wu and Tesh 1989). In this respect, infecting natural hosts with oocysts from distant locations could be an interesting approach, as they seem to produce higher mortality on hosts with which they have not co-evolved (Sulaiman 1992, Tseng 2007). It is to be noted that field surveys and studies to assess infection rates, distribution, and seasonality have mainly focused on mosquito gregarines (*Ascogregarina*) and, due to the scarcity of these studies in sand fly gregarines (*Psychodiella*), results related with the impact of these parasites on their host in the field remain inconclusive. Given the fact that we recently reported the identification of *Ps. chagasi* sequences in *Lu. longipalpis* from Posadas (McCarthy et al. 2011) and that the Argentine *Lu. longipalpis* population is significantly differentiated from Brazilian populations (Salomon et al. 2010), we were interested in developing a straightforward and sensitive assay to analyse the incidence of natural gregarine infections in *Lu. longipalpis* from this location.

In this context, light microscopy analysis during routine taxonomic identification of 59 female *Lu. longipalpis* captured in Posadas during December, 2010 (Acardi et al. 2010, Fernandez et al. 2010, Salomon et al. 2010, Santini et al. 2010, Santini et al., 2012), revealed that six females showed *Ps. chagasi* infection (up to 100 oocysts *per* female) (Figure 2A.1, Table 2). Given the fact that oocysts were found in adult *Lu. longipalpis*, this confirmed that the gregarine was completing its life cycle and, since the adults were females, the parasite was likely being transmitted to the insect's offspring. The proportion of naturally infected female *Lu. longipalpis* captured during the mentioned sampling period (10%), as determined by microscopy analysis, was comparable to what has previously been described for *Lu. longipalpis* (Adler and Mayrink, 1961).

Microscopy has been the main method of choice for the diagnosis of natural sand fly gregarine infections (Adler and Mayrink 1961, Coelho and Falcao 1964, Lewis et al. 1970, Brazil and Ryan 1984). Nevertheless, correct identification of *Psychodiella* species and the level of detection by microscopy depend on the experience of the microscopist and the time spent examining each slide. To overcome some of the limitations of this method, we developed a PCR-based assay for the diagnosis of *Psychodiella* sp. in *Lu. longipalpis*, and the *in silico* and *in vitro* stages of validation indicated that the *Psy*F-*Psy*R primer pair was highly sensitive and specific at the genus level. Furthermore, during the *in vitro* validation, we obtained robust and specific amplifications when using a 1:100 dilution of the original cDNA sample, where 100 sand flies had been processed and only four *Ps. chagasi* sequences had been identified by pyrosequencing, together with other numerous apicomplexan species (McCarthy et al. 2011) (Figure 1.C).

Nonetheless, to maximize the value of the PCR assay, it was important to couple high sensitivity and specificity of the designed primers with a simple DNA extraction method. In the latter case, DNA extraction protocols for phlebotomine sand flies (de Pita-Pereira et al. 2005, Acardi et al. 2010) are more appropriate for widescale analyses than the protocol we used in our previous study (total RNA extraction and cDNA amplification) (McCarthy et al. 2011). Nevertheless, subsequent amplification and detection can be affected by extraction efficiency, background matrix of genomic DNA and other biomolecules, such as pigments or proteins. To assess this aspect, DNA was extracted from field-captured sand flies and the expected DNA fragment was only amplified in the infected female pools (SA1.1 and SA1.2), confirming the robustness and specificity of the assay. The amplified DNA fragment was then sequenced and subsequent BLASTN analysis (Altschul et al. 1990)

indicated it was homologous to the *Ps. chagasi* SSU rRNA gene (Figure 2.A.2). Moreover, to our knowledge this is the first study in which gregarine sequences have successfully been amplified from total DNA extracted from a naturally infected adult host, in contrast to previous studies where gregarine sequences were directly amplified from gregarine DNA extracts (Carreno et al. 1999, Leander et al. 2003, Votypka et al. 2009, Lantova et al. 2010), a particularly significant advantage for field surveys. The *A. taiwanensis* genome has been estimated at 20 Mb (Templeton et al. 2010). Following Acardi et al. (2010), assuming that the total DNA content of *Ps. chagasi* is similar to that of *A. taiwanensis* given their phylogenetic relatedness (approximately 22 femtograms *per* genome), and considering that the detection limit of the assay was calculated in 0.1 femtograms, we estimate that this PCR-assay can easily detect as little as one sand fly gregarine genome per sand fly, which would make it a more sensitive diagnosis method than conventional microscopy.

Phylogenetic analysis of the sequence amplified during field validation, using different substitution models, showed that it formed a well-supported node with *Ps. chagasi* from *Lu. longipalpis* (Figure 2B). This confirmed our previous *in silico* analysis, which indicated that the ~200 bp region targeted by the diagnostic primers (*Psy*F and *Psy*R) was appropriate for identifying *Psychodiella* sp. (Figure 1B). This was particularly meaningful, considering that the phylogenetic position of *Psychodiella* sp. within the Apicomplexa has previously been determined using full-length SSU rDNA sequences amplified with universal primers (~1,750 bp) (Votypka et al. 2009, Lantova et al., 2010).

In summary, we describe the first PCR-based assay for the detection and identification of natural sand fly gregarine infections in *Lu. longipalpis*. We designed diagnostic primers *Psy*F and *Psy*R, based on the *Ps. chagasi* sequences that we previously identified in *Lu. longipalpis* from Posadas (McCarthy et al. 2011) that amplify a fragment of the *Psychodiella* SSU rRNA gene (Figure 1A). The specificity and sensitivity of the diagnostic primers was initially verified in PCR amplifications using the original cDNA samples (McCarthy et al. 2011) as template (Figure 1C). We then performed a field validation and confirmed the suitability of this PCR-based assay for identifying natural *Psychodiella* sp. infections in *Lu. longipalpis* (Figure 2). Namely, the DNA extraction process did not interfere with amplification and detection, the primers were highly specific when they were used to amplify total DNA extracted from an environmental source, and the assay proved to be extremely sensitive (estimated in one parasite genome per sand fly). The proportion of gregarine infection in field-caught female *Lu. longipalpis* from Posadas, as determined by light microscopy analysis, was comparable to what has previously been recorded for this sand fly (10%) (Adler and Mayrink 1961). Nevertheless, given the limitations of this method for the detection of gregarine infections and the high sensitivity and specificity of the PCR-based assay we developed, studies are underway to further analyse the incidence of this gregarine parasite in *Lu. longipalpis* from Posadas. Furthermore, this diagnostic assay, which is straightforward, low cost and not time consuming, has the potential to expedite field surveys, enabling the detection of sand fly gregarine infections, even at low levels, and allowing the screening of large numbers of samples in a reproducible manner.

Acknowledgments

This research was supported by Agencia Nacional de Promoción Científica y Tecnológica (PICT PRH 112) and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 0294) grants to CBM. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We are very grateful to Drs. Lucie Lantová and Jan Votýpka for their invaluable assistance in the identification of sand fly gregarine parasites, to Dr. Nagila F. C. Secundino for kindly providing the *P. falciparum* DNA, and to Dr. Luis Diambra for critically reviewing the manuscript. We also thank anonymous reviewers for helpful suggestions and corrections on a previous draft of the manuscript. We gratefully acknowledge Dra. Lilian Tartaglino from the Posadas Municipality Quality of Life Department and members of the Vector Laboratory (Secretaría de Calidad de Vida, Municipalidad de Posadas) for their support, logistics, and interest in the problems of the region; the neighbors of the city of Posadas for opening their houses; and the Research Network for Leishmaniasis in Argentina (Red de Investigación de Leishmaniasis en Argentina, REDILA), of which we are all members.

REFERENCES CITED

Acardi, S.A., D.J. Liotta, M.S. Santini, C.M. Romagosa, and O.D. Salomon. 2010. Detection of *Leishmania infantum* in naturally infected *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae) and *Canis familiaris* in Misiones, Argentina: the first report of a PCR-RFLP and sequencing-based confirmation assay. Mem. Inst. Oswaldo Cruz 105: 796-799.

Adler, S. and W. Mayrink. 1961. A gregarine, *Monocystis chagasi* n. sp., of *Phlebotomus longipalpis*. Remarks on the accessory glands of *P. longipalpis*. Rev. Inst. Med. Trop. Sao Paulo 3: 230-238.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Andrade Filho, J.D., E.A. Galati, and A.L. Falcao. 2003. Redescription of *Nyssomyia intermedia* (Lutz & Neiva, 1912) and *Nyssomyia neivai* (Pinto, 1926) (Diptera: Psychodidae). Mem. Inst. Oswaldo Cruz 98: 1059-1065.

Carreno, R.A., D.S. Martin, and J.R. Barta. 1999. *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. Parasitol. Res. 85: 899-904.

Coelho, M.d.V. and A.L. Falcao. 1964. Aspects of the life-cycle of "*Monocystis chagasi*" Adler and Mayrink, 1961. Revist. Brasileira Biol. 24: 417-421.

Comiskey, N.M., R.C. Lowrie, Jr., and D.M. Wesson. 1999a. Effect of nutrient levels and *Ascogregarina taiwanensis* (Apicomplexa: Lecudinidae) infections on the vector competence of *Aedes albopictus* (Diptera: Culicidae) for *Dirofilaria immitis* (Filarioidea: Onchocercidae). J. Med. Entomol. 36: 55-61.

Comiskey, N.M., R.C. Lowrie, Jr., and D.M. Wesson. 1999b. Role of habitat components on the dynamics of *Aedes albopictus* (Diptera: Culicidae) from New Orleans. J. Med. Entomol. 36:

313-320.

Correa Antonialli, S.A., T.G. Torres, A.C. Paranhos Filho, and J.E. Tolezano. 2007. Spatial analysis of American visceral leishmaniasis in Mato Grosso do Sul State, Central Brazil. J. Infect. 54: 509-514.

de Pita-Pereira, D., C.R. Alves, M.B. Souza, R.P. Brazil, A.L. Bertho, A. de Figueiredo Barbosa, and C.C. Britto. 2005. Identification of naturally infected *Lutzomyia intermedia* and *Lutzomyia migonei* with *Leishmania* (Viannia) *braziliensis* in Rio de Janeiro (Brazil) revealed by a PCR multiplex non-isotopic hybridisation assay. Trans. R. Soc. Trop. Med. Hyg. 99: 905-913.

Dougherty, M.J. and R.D. Ward 1991. Methods of reducing *Ascogregarina chagasi* parasitaemia in laboratory colonies of *Lutzomyia longipalpis*." Parassitologia 33 (Suppl): 185-191.

Feliciangeli, M.D., O. Delgado, B. Suarez, and A. Bravo. 2006. *Leishmania* and sand flies: proximity to woodland as a risk factor for infection in a rural focus of visceral leishmaniasis in west central Venezuela. Trop. Med. Int. Hlth. 11: 1785-1791.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791.

Fernandez, M.S., O.D. Salomon, R. Cavia, A.A. Perez, S.A. Acardi, and J.D. Guccione. 2010. *Lutzomyia longipalpis* spatial distribution and association with environmental variables in an urban focus of visceral leishmaniasis, Misiones, Argentina. Acta Trop. 114: 81-87.

Gascuel, O. 1997. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. Mol. Biol. Evol. 14: 685-695.

Grimaldi, G., Jr., R.B. Tesh, and D. McMahon-Pratt. 1989. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. Am. J. Trop. Med. Hyg. 41: 687-725.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT." Nucl. Acids. Symp. Ser. 41: 95-98.

Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22: 160-174.

Horak, E. and J.L. Cheype. 2007. *Entoloma dragonosporum* (Singer) E. Horak et *E. luteosplendidum* sp. nov: Deux espèces remarquables de la Guyane française. Bull. trimestriel Soc. Mycolog. France 123: 251-262.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.

Lantova, L., K. Ghosh, M. Svobodova, H.R. Braig, E. Rowton, P. Weina, P. Volf, and J. Votypka. 2010. The life cycle and host specificity of *Psychodiella sergenti* n. sp. and *Ps. tobbi* n. sp. (Protozoa: Apicomplexa) in sand flies *Phlebotomus sergenti* and *Ph. tobbi* (Diptera: Psychodidae). J. Invertebr. Pathol. 105: 182-189.

Lantova, L., M. Svobodova, and P. Volf. 2011. Effects of *Psychodiella sergenti* (Apicomplexa, Eugregarinorida) on its natural host *Phlebotomus sergenti* (Diptera, Psychodidae). J. Med. Entomol. 48: 985-990.

Lantova, L. and P. Volf 2012. The development of *Psychodiella sergenti* (Apicomplexa: Eugregarinorida) in *Phlebotomus sergenti* (Diptera: Psychodidae). Parasitology 139: 726-734.

Leander, B.S., R.E. Clopton, and P.J. Keeling. 2003. Phylogeny of gregarines (Apicomplexa) as inferred from small-subunit rDNA and beta-tubulin. Int. J. Syst. Evol. Microbiol. 53: 345-354.

Levine, N.D. (1985). Phylum Apicomplexa Levine 1970. In: J.J. Lee, S.H. Hutner and E.C. Bovee (eds.). *An Illustrated Guide to the Protozoa.* pp. 322-374. Society of Protozoologists. Lawrence, KS.

Lewis, D., R. Lainson, and J. Shaw. 1970. Determination of parous rates in phlebotomine sandflies, with special reference to Amazon species. Bull. Entomol. Res. 60: 209-219.

Lins, R.M., S.G. Oliveira, N.A. Souza, R.G. de Queiroz, S.C. Justiniano, R.D. Ward, C.P. Kyriacou, and A. A. Peixoto. 2002. Molecular evolution of the cacophony IVS6 region in sandflies. Insect Mol. Biol. 11: 117-122.

McCarthy, C.B., L.A. Diambra, and R.V. Rivera Pomar. 2011. Metagenomic analysis of taxa associated with *Lutzomyia longipalpis*, vector of visceral leishmaniasis, using an unbiased high-throughput approach. PLOS Negl. Trop. Dis. 5: e1304.

Ostrovska, K., A. Warburg, and J. Montoya-Lerma. 1990. *Ascogregarina saraviae* n. sp. (Apicomplexa: Lecudinidae) in *Lutzomyia lichyi* (Diptera: Psychodidae). J. Protozool. 37: 69-70.

Perkins, F.O., J.R. Barta, R.E. Clopton, M.A. Peirce, and S.J. Upton. 2000. Phylum Apicomplexa (Levine, 1970. In: J.J. Lee, G.F. Leedale, and P. Bradbury (eds.) *Illustrated Guide to the Protozoa.* 2nd ed. pp. 190-369. Lawrence Kansas, Allen Press.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25: 1253-1256.

Posada, D. and T.R. Buckley. 2004. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. Syst. Biol. 53: 793-808.

Salomon, O.D., A.S. Araki, J.G. Hamilton, S.A. Acardi, and A.A. Peixoto. 2010. Sex pheromone and period gene characterization of *Lutzomyia longipalpis* sensu lato (Lutz & Neiva) (Diptera: Psychodidae) from Posadas, Argentina. Mem. Inst. Oswaldo Cruz 105: 928-930.

Santini, M.S., M.S. Fernandez, A.A. Perez, A.E. Sandoval, and O.D. Salomon. 2012. *Lutzomyia longipalpis* abundance in the city of Posadas, northeastern Argentina: variations at different spatial scales. Mem. Inst. Oswaldo Cruz 107: 767-771.

Santini, M.S., O.D. Salomon, S.A. Acardi, E.A. Sandoval, and L. Tartaglino. 2010. *Lutzomyia longipalpis* behavior and control at an urban visceral leishmaniasis focus in Argentina. Rev. Inst. Med. Trop. Sao Paulo 52: 187-191.

Service, M. 1985. Some ecological considerations basic to the biocontrol of Culicidae and other medically important insects. *Integrated Mosquito Control Methodologies.* M. Laird (ed.). London, Academic Press. 2**:** 9-30.

Sharma, U. and S. Singh. 2008. Insect vectors of *Leishmania*: distribution, physiology and their control. J. Vector Borne Dis. 45: 255-272.

Shortt, H.E. and C.S. Swaminath. 1927. *Monocystis mackiei* n. sp. parasitic in *Phlebotomus argentipes*. Ann. Brun. Indian J. Med. Res. 15: 539-553.

Sudia, W.D. and R.W. Chamberlain 1962. Battery operated light

trap, an improved model. Mosq. News 22: 126-129.

Sulaiman, I. 1992. Infectivity and pathogenicity of *Ascogregarina culicis* (Eugregarinida: Lecudinidae) to *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 29: 1-4.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731-2739.

Templeton, T.J., S. Enomoto, W.J. Chen, C.G. Huang, C.A. Lancto, M.S. Abrahamsen, and G. Zhu. 2010. A genome-sequence survey for *Ascogregarina taiwanensis* supports evolutionary affiliation but metabolic diversity between a gregarine and *Cryptosporidium*. Mol. Biol. Evol. 27: 235-248.

Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22: 4673-4680.

Tseng, M. 2004. Sex-specific response of a mosquito to parasites and crowding. Proc. Biol. Sci. 271 (Suppl. 4): S186-188.

Vaux, D.L. 1992. Rapid recovery of DNA from agarose gels. Trends Genet 8: 81.

Votypka, J., L. Lantova, K. Ghosh, H. Braig, and P. Volf. 2009. Molecular characterization of gregarines from sand flies (Diptera: Psychodidae) and description of *Psychodiella* n. g. (Apicomplexa: Gregarinida). J. Eukaryot. Microbiol. 56: 583-588.

Warburg, A. and K. Ostrovska. 1991. Host-parasite relationships of *Ascogregarina chagasi* (Eugregarinorida, Aseptatorina, Lecudinidae) in *Lutzomyia longipalpis* (Diptera: Psychodidae). Int. J. Parasitol. 21: 91-98.

Wu, W.K. and R.B. Tesh. 1989. Experimental infection of Old and New World phlebotomine sand flies (Diptera: Psychodidae) with *Ascogregarina chagasi* (Eugregarinorida: Lecudinidae). J. Med. Entomol. 26: 237-242.

Young, D. and M. Duncan. 1994. Guide to the identification and geographic distribution of *Lutzomyia* sand-flies in Mexico,West Indies, Central and South America (Diptera: Psychodidae). Mem. Am. Entomol. Inst. 54: 1-881.

Young, D.G. and D.J. Lewis. 1977. Pathogens of Psychodidae (phlebotomine sand flies). Bull. Wld. Hlth. Org. 55 (Suppl.) 1: 9-17.