POSTGLACIAL DISPERSAL OF *PHLEBOTOMUS PERNICIOSUS* INTO FRANCE

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Summary:

Phlebotomus perniciosus was identified morphologically in samples from France and northeast Spain, and individuals were then characterized at three polymorphic isoenzyme loci (by isoelectrofocusing) and at the mitochondrial DNA locus (by comparative DNA sequence analysis of a fragment of the Cytochrome b gene). The four polymorphic loci gave conflicting patterns of population relationships, which can be explained by hypothesizing different amounts of gene introgression at each locus when two distinctive lineages met in southern France or northeast Spain after isolation in southern Italy and Spain during the Pleistocene Ice Ages. *P. perniciosus* is an important vector of *Leishmania infantum* and so these population differentiation studies are relevant for predicting the emergence and spread of leishmaniasis in relation to environmental changes, including climate.

KEY WORDS : *Phlebotomus perniciosus*, isoenzyme, mitochondrial lineage, phylogeography, postglacial dispersal, *Leishmania infantum*, France.

Résumé : DISPERSION POSTGLACIAIRE DE *PHLEBOTOMUS PERNICIOSUS* EN FRANCE

Phlebotomus perniciosus *est identifié morphologiquement sur des échantillons de France et du nord-est de l'Espagne. Des individus sont caractérisés aux loci de trois systèmes enzymatiques polymorphes (par isoélectrofocalisation) et au locus de l'ADN mitochondrial (par analyse comparative des séquences de l'ADN d'un fragment du gène du cytochrome b). La comparaison de ces quatre locus polymorphes révèle des contradictions dans les relations entre les populations étudiées. Ceci peut s'expliquer par l'hypothèse de niveaux différents d'introgression à chaque locus lorsque deux lignées isolées pendant les glaciations du Pléistocène en Italie du sud et en Espagne se sont rencontrées dans le sud de la France ou le nord-est de l'Espagne.* P. perniciosus *est un vecteur important de* Leishmania infantum *et les études sur la différenciation des populations sont utiles dans la prévision de l'émergence et de l'extension des leishmanioses en relation avec les modifications de l'environnement dont les changements climatiques.*

MOTS CLÉS : Phlebotomus perniciosus, *isoenzyme, lignée mitochondriale, phylogéographie, dispersion postglaciaire,* Leishmania infantum, *France.*

INTRODUCTION

The haematophagous females of *Phlebotomus (Larroussius) ariasi* Tonnoir, 1921 and *Phlebotomus (Larroussius) perniciosus* Newstead, 1911 are the phlebotomine sandflies (Diptera: Psychodidae) that have been incriminated as vectors of *Leishmania infantum* Nicolle, 1908 in Mediterranean France (Rioux & Golvan, 1969; Killick-Kendrick & Rioux, 1981; Killick-Kendrick, 1990). Domestic dogs and wild foxes are the reservoir hosts of *L. infantum*, which is the causative agent of both cutaneous and visceral leishmaniasis in humans and canines (Rioux & Lanotte, 1990).

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Phlebotomus perniciosus is the more widespread of the two vectors in non-Mediterranean France, having been recorded in the suburbs of Paris (Rioux & Golvan, 1969) and incriminated as the probable transmitter of *L. infantum* among dogs in Touraine, where *P. ariasi* was rare in the early 1970s (Houin *et al.*, 1975), and in the north of Auvergne, where *P. ariasi* has not been recorded (Pesson *et al.*, 1985).

There had been some confusion about the morphological characters diagnostic for *P. perniciosus* (Benabdennbi *et al.*, 1999), but this has recently been resolved by Pesson *et al.* (2004), who also provided isoenzyme and mitochondrial (mt) DNA characters for distinguishing the two geographical lineages (typical and Iberian) first reported by Esseghir *et al.* (1997, 2000). The typical lineage is so called because it contains a population from Malta, the species' type locality.

Phlebotomus perniciosus requires Mediterranean-like summers for adult activity and warm winters for the survival of diapausing larvae (Rioux & Golvan, 1969; Ready & Croset, 1980), and so it could not have survived in western Europe during all Pleistocene Ice Ages except in southern Spain and Italy, where the two lineages possibly

evolved (Esseghir *et al.*, 2000). In the present report, isoenzyme and mtDNA markers (Avise, 2000) are used for the first time to characterize French populations of *P. perniciosus*, in order to investigate their postglacial origins.

MATERIALS AND METHODS

STUDY AREAS

The locations from which *P. perniciosus* was sampled are shown in Figure 1. The bioclimate data in French locations comes from CNRS vegetation maps (Corillion, 1973; Dupias, 1971; Ozenda, 1961). The natural vegetation is given, not land cover.

Touraine region, France
Population code: F_IL_CMP. Cinq-Mars-la-Pile (47° 21' N, 0° 28' E), département of Indre-et-Loire; altitude 40 m a.s.l.; oceanic climate, with slight Mediterranean influence; Loire valley; natural forests of *Fraxinus excelsior* and oak, dominated by the pedunculate oak, *Quercus robur*; northern limit of the pubescent oak, *Quercus pubescens*.

Auvergne region, France
Population code: F_PD_MEZ. Mezel (45° 45' N, 3° 13' E), département of Puy-de-Dôme; altitude 360 m a.s.l.; continental climate with Mediterranean influence; volcanic mound in the Allier valley; sub-Mediterranean vegetation with pubescent oak, *Q. pubescens*.
Population code: F_PD_VEG. Les Végheants (45° 43' N, 3° 22' E), département of Puy-de-Dôme; altitude 470 m a.s.l.; continental climate with Mediterranean influence; hills at edge of the Allier valley; woodland dominated by the pedunculate oak, *Q. robur*.

Côte d'Azur region, France
Population code: F_AM_BEA, sampled in 1988, 1998 and 2001. Beaulieu-sur-Mer (43° 42' N, 7° 19' N), département of Alpes-Maritimes; altitude 20 m a.s.l.; subhumid Mediterranean climate; hilly zone of the mediterranean coast; Mediterranean vegetation, including olive trees and the carob, *Ceratonia silica*.

Languedoc region, France
Population code: F_G_SUM. Sumène (43° 58' N, 3° 43' E), département of Gard; altitude 373 m a.s.l.; humid Mediterranean climate; southern calcareous foothills of

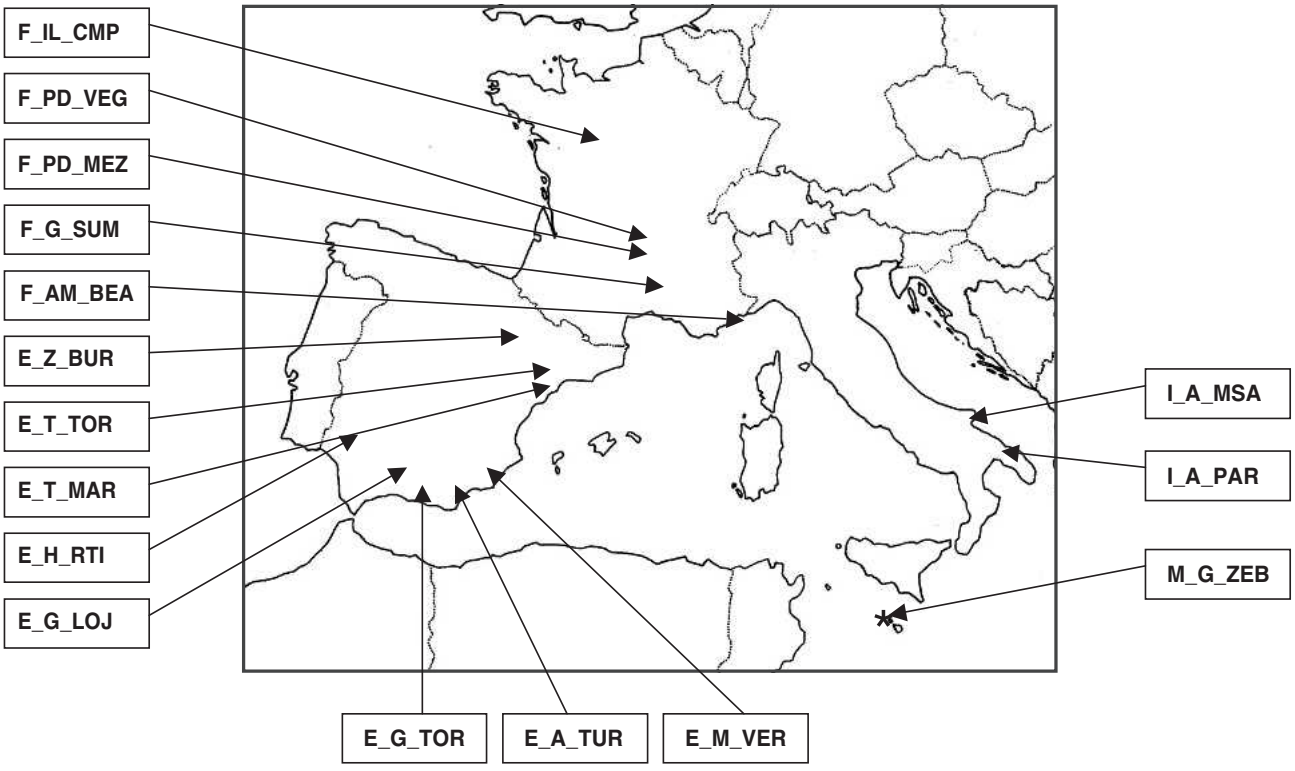

Fig. 1. – Geographical locations of the sampled populations of *P. perniciosus* and (*) its type-locality.
Key to populations by country, region, locality: F_IL_CMP = France, Touraine (Indre-et-Loire), Cinq-Mars-la-Pile; F_PD_MEZ = France, Auvergne (Puy de Dôme), Mezel; F_PD_VEG = France, Auvergne (Puy de Dôme), Les Végheants; F_AM_BEA = France, Côte d'Azur (Alpes Maritimes), Beaulieu; F_G_SUM = France, Languedoc (Gard), Sumène; E_T_TOR = Spain, Catalonia (Tarragona), Torroja del Priorat; E_T_MAR = Spain, Catalonia (Tarragona), Margalef; E_Z_BUR = Spain, Aragon (Zaragoza), El Burgo de Ebro; E_M_VER = Spain, Murcia, Verdolay; E_A_TUR = Spain, Andalucia (Almería), Turre; E_G_LOJ = Spain, Andalucia (Granada), Loja; E_G_TOR = Spain, Andalucia (Granada), Torvizcón; E_H_RTI = Spain, Andalucia (Huelva), Rio Tinto; M_G_ZEB = Malta, Gozo island, Zebbug; I_A_MSA = Italy, Apulia, Monte Sant'Angelo; I_A_PAR = Italy, Apulia, Parabita.

the Cévennes mountains; Mediterranean vegetation, with mixed woodlands of evergreen holm oak, *Q. ilex*, and pubescent oak, *Q. pubescens*.

Catalonia region, Spain
Population codes: E_T_TOR and E_T_MAR, respectively. Torroja del Priorat (41° 13' N, 0° 49' E) altitude 332 m a.s.l., and Margalef (41° 16' N, 0° 45' E) altitude 379 m a.s.l., Tarragona province; sub-humid Mediterranean climate; southern foothills of the Pyrenees mountains; Mediterranean vegetation, with mixed woodlands of evergreen holm oak, *Q. ilex*, and pubescent oak, *Q. pubescens*.

Aragon region, Spain
Population code: E_Z_BUR. El Burgo de Ebro (41° 34' N, 0° 43' W), Zaragoza province; altitude 172 m a.s.l.; semi-arid Mediterranean climate; Ebro valley; steppe vegetation of the Monegros.

Other Mediterranean regions
For comparison, published data (Pesson *et al.*, 2004) was used for populations of *P. perniciosus* from four provinces in southern Spain (Population codes: E_M_VER for Verdolay in Murcia; E_A_TUR for Turre in Almería; E_G_TOR for Torvizcón and E_G_LOJ for Loja, both in Granada; E_H_RTI for Rio Tinto in Huelva), one location in Malta (Population code: M_G_ZEB for Zebbug on the island of Gozo) and two locations in Italy (Population codes: I_A_MSA for Monte-Sant'Angelo and I_A_PAR for Parabita, both in Apulia).

COLLECTION AND IDENTIFICATION OF SPECIMENS

Sandflies were captured overnight in miniature CDC light traps (Sudia & Chamberland, 1962) and immediately conserved in liquid nitrogen or in analytical grade ethanol at −20° C if isoenzymes were not to be characterized. Sandflies were identified based on external and internal morphological characters of the head and genitalia (Léger *et al.*, 1983; Gil Collado *et al.*, 1989; Benabdennbi *et al.*, 1999; Pesson *et al.*, 2004), which were slide-mounted in Berlese fluid or Marc André solution following dissection with sterilized forceps and microneedles. Dissections were carried out in a room distant from the molecular biology laboratory, following the protocols of Testa *et al.* (2002) to reduce the risk of Polymerase Chain Reaction (PCR) carryover. The dissected thorax and the anterior abdomen were used, separately or combined, to extract proteins or total DNA.

ISOENZYME CHARACTERIZATION OF *P. PERNICIOSUS*

In Strasbourg, dissected bodyparts (thorax and/or the anterior abdomen) of individual sandflies were homogenized in 45 µl of distilled water using a glass rod, and 10 µl of this suspension of total proteins was used to characterize the isoenzymes at each locus by isoelectrofocusing. This was carried out in ultrathin agarose gels (Pharmacia™ mould) with the ampholyte at pH 4.0-6.5, following the protocols described by Pesson *et al.* (1991). Pesson *et al.* (2004) found only three out of 11 loci to be sufficiently polymorphic for studying the population differentiation of *P. perniciosus*, and all three were used in the current investigation, namely: hexokinase (HK, E.C.2.7.1.1), glucosephosphate isomerase (GPI, E.C.5.3.1.9) and phosphoglucomutase (PGM, E.C.5.4.2.2). The alleles, visualized as coloured bands on the gels, were numbered chronologically for *P. perniciosus* (and other species of the subgenus *Larroussius*; Pesson *et al.*, 2004): for HK, allele 1 = pHi 5.60, allele 2 = pHi 5.49, allele 3 = pHi 5.36, allele 5 = pHi 5.58, allele 7 = pHi 5.22; for GPI, allele 1 = pHi 5.30, allele 2 = pHi 5.64, allele 3 = pHi 5.00, allele 4 = pHi 6.19, allele 5 = pHi 5.09, allele 6 = pHi 5.43, allele 8 = pHi 5.60, allele 9 = pHi 5.80; for PGM, allele 1 = pHi 4.93, allele 2 = pHi 5.24, allele 3 = pHi 5.10, allele 4 = pHi 5.34, allele 5 = pHi 5.47, allele 6 = pHi 5.15, allele 7 = pHi 5.29, allele 8 = pHi 5.45.
The allele frequencies, tests for deviation of genotype frequencies from Hardy Weinberg equilibrium, and UPGMA phenetic analyses based on Nei's genetic distances and F_{ST} values were calculated using BIOSYS-2 (Swofford & Selander, 1981) and PHYLIP version 3.6a2 (Felsenstein, 1989). In addition, GENEPOP (Raymond & Rousset, 1995) was used to test for genotypic linkage disequilibrium and to estimate F_{ST} values between each pair of populations.

DNA ISOLATION, PCR AMPLIFICATION AND COMPARATIVE SEQUENCING OF MITOCHONDRIAL DNA OF *P. PERNICIOSUS*

In London, genomic DNA was extracted from the thorax and/or the anterior abdomen of individual sandflies (Ready *et al.*, 1991). The CB3 fragment of mtDNA (545 base pairs (bp) including primers) was amplified by PCR using the primer pair CB3-FC/N1N-FA (Esseghir *et al.*, 1997), to obtain the 3′ end of the gene Cytochrome b (Cyt b).
A 50 µl PCR reaction mixture consisted of 1 × Promega buffer, 1.5 mM $MgCl_2$, 60 µM each dNTP, 0.5 µM each of the two primers and 1.5 units of Taq DNA polymerase (Promega). DNA amplification was performed in a Perkin Elmer Thermocycler 9700 with the following amplification cycles: denaturation at 94° C for three min; five cycles of denaturation at 94° C for 30 s, annealing at 40° C for 30 s, extension at 72° C for 90 s; followed by 30-35 cycles of denaturation at 94° C for 30 s, annealing at 44° C for 30 s, extension at 72° C for 90 sec; and a final extension at 72° C for 10 min. The size and quantity of the amplified DNA fragments

were determined by fractionation in 1.5 % agarose gels along with DNA standards (Promega PCR Markers G316A). The DNA in each excised gel fragment was purified with glassmilk (Geneclean II Kit, BIO 101 Inc) and each strand directly sequenced using one of the PCR primers (3.2 pmoles) and the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (versions 2.0 and 3.1, PE Applied Biosystems). The extension products were purified by ethanol precipitation and their sequences were resolved using an ABI 373A or 377 automated system, all according to ABI protocols (PE Applied Biosystems). The sequences obtained were then edited and aligned with database sequences using Sequencher™ software (Gene Codes Corp.) to identify haplotypes (*i.e.* unique sequences) and to permit their phylogenetic analysis using PAUP* software (Swofford, 2002).

RESULTS

Morphological identification of *P. perniciosus*

Diagnostic morphological characters (Pesson *et al.*, 2004) were used to select *P. perniciosus sensu stricto* for isoenzyme and mtDNA characterization. All females had a single spherical and basal dilatation on each of the two individual spermathecal ducts, and all males had bifurcated aedeagi. The mean number of coxite setae in French populations was also typical of males of *P. perniciosus* (range: 8-19; Table I), not of *P. longicuspis sensu stricto* (range: 21-31) (Pesson *et al.*, 2004).

Isoenzyme characterisation of *P. perniciosus*

All 17 *P. perniciosus* populations (Table II) were in Hardy Weinberg (HW) equilibrium except at the GPI locus in three populations (F_PD_MEZ Mezel, E_Z_BUR El Burgo de Ebro and E_G_TOR Torvizcon). These departures from equilibrium were confirmed in multilocus and multipopulation testing, and they were due to the presence in a few individuals of two rare GPI alleles (05 and 06) with heterozygote deficit for the most common allele (01). Apparent departures from panmixis are often found for a small number of populations in such analyses, and in this case their biological significance is not clear.

There was no significant linkage disequilibrium except for two populations (E_G_LOJ Loja PGM-HK, $P = 0.038$ and E_H_RTI Rio Tinto GPI-PGM, $P = 0.046$), but this is not believed to have any biological significance because the test for each locus pair across all populations was not significant ($P > 0.45$).

Allele and genotype frequencies at the three polymorphic loci differed little among all five French locations, which were characterized by a wide range of bioclimates. In Beaulieu, Côte d'Azur (F_AM_BEA), little variation was observed between the three temporal populations sampled from 1988 to 2001 (Table II). Comparing the populations from different countries: PGM was not discriminating; HK unambiguously linked the French with all the Spanish populations (frequency

Population	n	Min	Max	Mean + SD
France (F_IL_CMP)[1]	65	11	19	14.88 ± 1.75
France (Auvergne 1983)	30	11	17	13.63 ± 1.45
France (F_AM_BEA)	25	11	16	13.68 ± 1.14
Spain (E_T_TOR)	22	9	17	13.95 ± 2.26
Spain (E_Z_BUR)	30	9	16	12.83 ± 1.93
Spain (E_M_VER)	19	10	16	12.15 ± 1.71
Spain (E_A_TUR)	30	10	17	13.40 ± 1.35
Spain (E_H_RTI)	24	9	15	11.79 ± 1.69
Malta (types)	22	9	16	13.14 ± 1.69
Malta (M_G_ZEB)	29	10	17	13.45 ± 1.76
Italy (I_A_MSA)	30	10	16	12.83 ± 1.26

Table I. – The mean number of coxite hairs for populations of *P. perniciosus*. n = number of males sampled; Min = minimum number of hairs; Max = maximum number of hairs; SD = standard deviation; [1] = new data; other data from Benabdennbi & Pesson (1998).

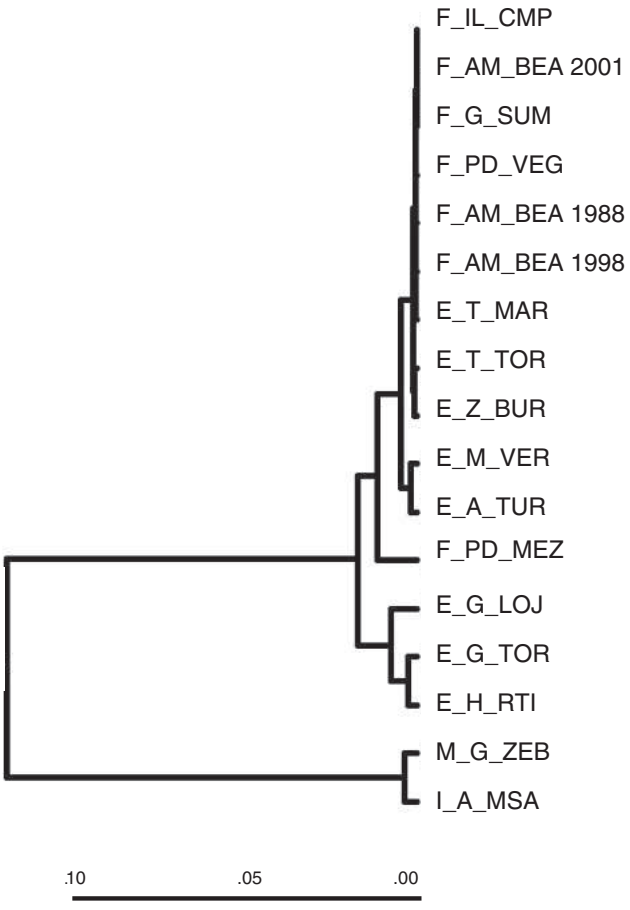

Fig. 2. – Cluster analysis by UPGMA of Nei's genetic distances among 17 populations of *P. perniciosus*, calculated from allele frequencies at the three polymorphic isoenzyme loci (HK, GPI, PGM).

	Populations of *Phlebotomus perniciosus*																
Locus	**F_ IL_CMP**	**F_ PD_MEZ**	**F_ PD_VEG**	**F_ AM_BEA 1988**	**F_ AM_BEA 1998**	**F_ AM_BEA 2001**	**F_ G_SUM**	**E_ T_TOR**	**E_ T_MAR**	**E_ Z_BUR**	**E_ M_VER**	**E_ A_TUR**	**E_ G_LOJ**	**E_ G_TOR**	**E_ H_RTI**	**M_ G_EZB**	**I_ A_MSA**
HK																	
No.	83	23	31	19	34	8	8	45	16	34	21	18	13	98	22	63	119
1	1.000	1.000	1.000	0.974	0.956	1.000	1.000	0.989	0.969	1.000	0.929	0.889	0.885	0.929	1.000	0.413	0.458
2	0.000	0.000	0.000	0.026	0.044	0.000	0.000	0.000	0.031	0.000	0.071	0.111	0.115	0.066	0.000	0.587	0.517
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.000
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
P	–	–	–	*1*	*1*	–	–	*1*	*1*	–	*1*	*1*	*1*	*1*	–	*0.799*	*0.855*
GPI																	
No.	83	23	31	19	34	8	8	45	16	34	21	18	13	98	22	63	119
1	1.000	0.913	1.000	1.000	1.000	1.000	1.000	0.933	1.000	0.956	0.881	0.889	0.692	0.776	0.773	0.960	0.987
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.015	0.071	0.111	0.269	0.153	0.205	0.106	0.008
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.048	0.000	0.038	0.015	0.023	0.008	0.000
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.016	0.000
5	0.000	0.065	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.020	0.000	0.000	0.000
6	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.000
9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004
P	–	*0.002*	–	–	–	–	–	*0.163*	–	*0.045*	*1*	*0.166*	*1*	*0.021*	*0.271*	*1*	*1*
PGM																	
No.	83	23	31	19	34	8	8	45	16	34	21	18	13	98	22	63	119
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004
2	1.000	0.826	0.984	1.000	0.985	1.000	1.000	0.967	0.969	0.985	0.976	1.000	0.885	0.980	0.977	0.897	0.900
3	0.000	0.174	0.016	0.000	0.015	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.024	0.000
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.031	0.000	0.024	0.000	0.115	0.005	0.023	0.063	0.013
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.000
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000	0.000	0.004
P	–	*0.098*	*1*	–	*1*	–	–	*1*	*1*	*1*	*1*	–	*0.120*	*1*	*1*	*0.498*	*1*

Table II. – Allelic frequencies at the three polymorphic isoenzyme loci characterized in 17 populations of *P. perniciosus*. Origins and their abbreviations are given in Figure 1. HK = hexokinase, GPI = glucosephosphate isomerase and PGM = phosphoglucomutase; P = probability of χ^2 value occurring by chance, when testing for deviation from Hardy-Weinberg expectations of genotype frequencies.

of allele 1 ≥ 0.885), and linked the Italian population with the Maltese (frequency of allele 1 ≤ 0.458); and, GPI grouped the French populations with those from northeast Spain (Catalonia and Zaragoza), Italy and Malta (frequency of allele 1 ≥ 0.913), rather than with those from southern Spain (frequency of allele 1 ≤ 0.889).

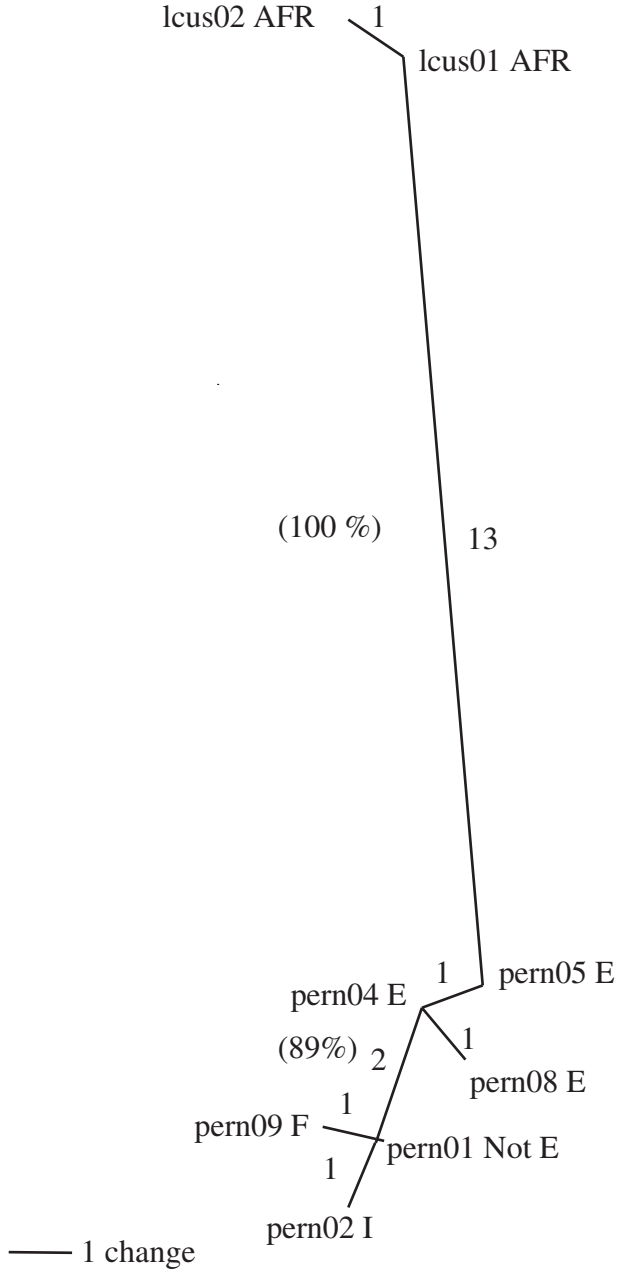

Fig. 3. – Phylogenetic relationships among all the CB3 haplotypes of mitochondrial Cyt b of *P. perniciosus* (pern) and *P. longicuspis* (lcus). Each haplotype is numbered and labelled with the country or region in which it has been found (E = Spain, F = France, I = Italy, Not E = Malta, Italy and France, AFR = northwest Africa; Table IV). The unrooted phylogram is the shortest tree generated by a parsimony analysis with a branch and bound search (PAUP*: Swofford, 2002). Each branch is marked with the number of nucleotide differences between a pair of haplotypes, and bootstrap values > 80 % are shown.

Populations of *P. perniciosus*	F_ IL_CMP	F_ PD_MEZ	F_ PD_VEG	F_ AM_BEA 1988	F_ AM_BEA 1998	F_ AM_BEA 2001	F_ G_SUM	E_ T_TOR	E_ T_MAR	E_ Z_BUR	E_ M_VER	E_ A_TUR	E_ G_LOJ	E_ G_TOR	E_ H_RTI	M_ G_EZB	I_ A_MSA
F_IL_CMP		0.010	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.003	0.006	0.030	0.011	0.015	0.134	0.104
F_PD_MEZ	0.2576	–	0.008	0.011	0.010	0.010	0.010	0.010	0.010	0.009	0.015	0.020	0.040	0.021	0.024	0.163	0.134
F_PD_VEG	0.0185	0.1040		0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.004	0.007	0.031	0.011	0.015	0.135	0.105
F_AM_BEA 1988	0.0530	0.0886	0.0026		0.000	0.000	0.000	0.001	0.000	0.001	0.003	0.005	0.030	0.011	0.016	0.124	0.095
F_AM_BEA 1988	0.0522	0.0958	0.0105	-0.0146		0.001	0.001	0.002	0.000	0.001	0.003	0.005	0.030	0.011	0.017	0.117	0.104
F_AM_BEA 2001	–	0.0375	-0.0306	-0.0271	-0.0153		0.000	0.001	0.000	0.000	0.003	0.006	0.030	0.011	0.015	0.134	0.104
F_G_SUM	–	0.0375	-0.0306	-0.0271	-0.0153	–		0.001	0.000	0.000	0.003	0.006	0.030	0.011	0.015	0.134	0.110
E_T_TOR	0.0402	0.0671	0.0099	0.0039	0.0149	-0.0231	-0.0231		0.001	0.001	0.002	0.006	0.024	0.008	0.011	0.141	0.110
E_T_MAR	0.0746	0.0587	0.033	-0.0174	-0.0152	-0.0248	-0.0248	-0.0047		0.001	0.003	0.006	0.029	0.012	0.016	0.123	0.095
E_Z_BUR	0.0348	0.0815	0.0014	-0.0029	0.0133	-0.0416	-0.0416	-0.0054	-0.0036		0.003	0.006	0.027	0.008	0.012	0.123	0.108
E_M_VER	0.1597	0.0557	0.0661	0.0315	0.0336	0.0142	0.0142	0.0091	0.0172	0.0286		0.002	0.016	0.004	0.008	0.118	0.091
E_A_TUR	0.2464	0.0761	0.1088	0.0523	0.0527	0.0312	0.0312	0.0401	0.0363	0.0604	-0.0174		0.015	0.004	0.009	0.102	0.076
E_G_LOJ	0.4806	0.1105	0.2648	0.1914	0.2211	0.1270	0.1270	0.1608	0.1487	0.2032	0.0475	0.0374		0.008	0.007	0.143	0.119
E_G_TOR	0.1199	0.0755	0.0799	0.0619	0.0703	0.0424	0.0424	0.0461	0.0559	0.0561	0.0031	-0.0001	0.0115		0.002	0.136	0.107
E_H_RTI	0.3489	0.0954	0.1835	0.1411	0.1600	0.0911	0.0911	0.0792	0.1156	0.1141	0.0230	0.0273	0.0115	-0.0032		0.171	0.138
M_G_ZEB	0.5180	0.3420	0.3991	0.3440	0.3567	0.3274	0.3274	0.3878	0.3226	0.3927	0.2834	0.2516	0.2605	0.3194	0.3581		0.003
I_A_MSA	0.4450	0.3486	0.3671	0.3222	0.3230	0.3216	0.3216	0.3586	0.3087	0.3631	0.2729	0.2379	0.2852	0.2982	0.3590	0.0087	

Table III. – Below diagonal: Fst estimated as in Weir & Cockerham (1984). Above diagonal: Nei (1972) genetic distance. Values calculated from the isoenzyme data at the three polymorphic loci for the 17 populations of *P. perniciosus*.

Nei's genetic distances (*D*) and F_{ST} values were calculated between all pairs of populations, using the combined data from all three polymorphic loci (Table III). Phenetic analysis of Nei's *D* values, which assumes no gene flow, indicated that there are two primary geographical clusters of *P. perniciosus* populations, Italy-plus-Malta and France-plus-Spain, as presented graphically in the UPGMA tree (Fig. 2). This topology is determined by the quantitative influence of HK. Within the France-plus-Spain cluster, there is a secondary cluster formed by the three populations from the southwest of Spain, reflecting the lower frequency (≤ 0.776) of GPI allele 1. The cluster analysis using F_{ST} values, which assumes possible gene flow, indicated the same geographic structure.

COMPARATIVE SEQUENCE ANALYSIS OF MITOCHONDRIAL CYT B OF *P. PERNICIOSUS*

The last 279 nucleotides of Cyt b were obtained for at least 10 individuals of *P. perniciosus* from four French populations (F_IL_CMP, F_PD_MEZ, F_PD_VEG, F_AM_BEA) and for six individuals of another population of this species from Catalonia region, northeast Spain (E_T_TOR). These new sequences were then aligned with those already obtained (Esseghir *et al.*, 1997; Pesson *et al.*, 2004) from western Mediterranean countries other than France. Haplotypes were identified (Table IV) by inspection of the input data matrix and the genetic distance matrix given by PAUP* (Swofford, 2002).

A phylogenetic analysis based on parsimony placed the haplotypes of all the new sequences in the *P. perniciosus* clade (Fig. 3), with a large genetic distance (13 fixed polymorphisms) separating it from the outgroup clade of *P. longicuspis* from northwest Africa; this branch had 100 % bootstrap support, based on 1,000 replicates. Within *P. perniciosus*, a well supported subclade (89 % bootstrap support, 1,000 replicates) contained all the haplotypes found in the populations from Malta (the type locality), Italy and France. This finding was reported, without detailed results or analysis, by Mahamdallie *et al.* (2003). Therefore, mitochondrially, the populations of *P. perniciosus* from France belonged to the typical lineage (Esseghir *et al.*, 1997, 2000), and not to the Iberian lineage, which contained the haplotypes found in the E_T_TOR population from northeast Spain (Fig. 3). Two fixed polymorphisms were diagnostic for the typical lineage (Table IV).

DISCUSSION

The results obtained by the characterization of isoenzymes and mitochondrial Cyt b appear to be contradictory for the populations of *P. perniciosus* from France and Catalonia region, northeast Spain. For the isoenzymes, most of the contradictory population structure was given by two loci. At the HK locus, the newly characterized populations from France and northeast Spain grouped with those from southern Spain, whereas at the GPI locus these new populations grouped with those from Italy and Malta (Tables II, III). Based on the cluster analysis for all three polymorphic isoenzyme loci, the populations from France were

	Character number in alignment[a]	Number of specimens with each halotype in different localities[b]										
		France				Spain					Malta	Italy
Species Lineage & Haplotype of CB3 Cyt b	1111111222 24557773446788455 6979482483578113628	F_ IL_CMP	F_ PD_MEZ	F_ PD_VEG	F_ AM_BEA	E_ T_TOR	E_ M_VER	E_ A_TUR	E_ G_TOR	E_ H_RTI	M_ G_ZEB	I_ A_PAR
P. longicuspis N African lineage												
lcus 01 AFR	CGTCCCCCTGGTCTGCTCA											
lcus 02 AFR	CGTCCCCCTGGTCTACTCA											
P. perniciosus Typical lineage												
pern 01 Not E	TAATTTTCCGACTCGAATG	22	12	11	9						2	1
per02 I	TAATATTCCGACTCGAATG											1
pern 09 F	TAATTTTTCGACTCGAATG				1							
P. perniciosus Iberian lineage												
pern 04 E	TAATTTTCCGATTCGATTG					5	6	6	9	14		
pern 05 E	TAATTTTCTGATTCGATTG									2		
pern 08 E	TAATTTTCCAATTCGATTG					1						

[a] Underlined nucleotides = synapomorphic characters diagnostic for lineages; [b] Underlined numbers = data from Esseghir *et al.* (1997) and Pesson *et al.* (2004).

Table IV. - Alignment of the haplotypes of the CB3 fragment of Cyt b (variant characters only) related to the specimens' origins.

most similar to those from Spain, not those from Italy and Malta (Fig. 2). In general, there was no linear relationship between genetic isolation and geographical distance, as evidenced (Fig. 2) by the placement of one French Auvergne population (F_PD_MEZ) amongst the southern Spanish populations rather than amongst the other French ones.

The isoenzyme cluster analysis conflicts with the mitochondrial results (Fig. 3), because the latter unequivocally places the populations from France with those from Italy and Malta, not with those from Spain. The contradictory results for the populations from France and northeast Spain can be explained by hypothesizing different levels of gene introgression following hybridization between the typical and Iberian Cyt b lineages of *P. perniciosus*. These two lineages were discovered by Esseghir *et al.* (1997, 2000) and Pesson *et al.* (2004), who recorded them from Morocco, Tunisia, Malta and Italy (the typical lineage), and from southern Spain (Iberian lineage). During the Pleistocene Ice Ages, *P. perniciosus* could have survived in western Europe, but only continuously in refugia in southern Spain and southern Italy, not alwways in France and further north where temperatures often would have been too low for adult activities in the summer (Rioux & Golvan, 1969) and for the survival of diapausing larvae in the winter (Ready & Croset, 1980). Intraspecific gene introgression could have occurred following postglacial dispersal from Mediterranean refugia, some 12,000 years ago for the last such possible event (Esseghir *et al.*, 2000). For temperate animals and plants, Hewitt (1996, 1999) proposed three broad patterns of postglacial dispersal into northern Europe from southern refugia. Analysing many of the same case studies, Taberlet *et al.* (1998) highlighted four main suturezones where lineages from different refugia often meet, including two in southern France. A precise dispersal pattern has not been hypothesized for *P. perniciosus* because too few populations have been characterized, but this sandfly requires Mediterranean summers and so it is unlikely to have followed all the colonization routes of temperate species.

The analysis of allelic variation at five polymorphic microsatellite DNA loci has demonstrated strong genetic differentiation between populations of *P. perniciosus* from northeast Spain (including E_T_TOR) and southern Spain (Aransay *et al.*, 2003), and the frequency distribution of GPI allele 1 in the current study (Tables II, III) is consistent with this. This conclusion might be strengthened by discovering additional polymorphic enzyme loci.

Benlarbi & Ready (2003) found no evidence that "infectious speciation" has promoted the genetic differentiation of the typical and Iberian Cyt b lineages of *P. perniciosus*, or of the two Iberian regional populations in northeast and southern Spain. Infection of two arthropod populations by different strains of endosymbiotic *Wolbachia pipientis* (Alpha-proteobacteria, Rickettsiales) can produce reproductive barriers that lead to "infectious speciation" (Wade, 2001). However, only one *Wolbachia* strain was found in *P. perniciosus* throughout its range (Benlarbi & Ready, 2003).

The current investigation of the origins of northern populations of *P. perniciosus* has epidemiological significance because, when integrated with ecological studies and GIS techniques, it will help predict the emergence and spread of leishmaniasis in relation to environmental changes, including climate (Ready, 2004).

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