GENETIC POLYMORPHISM IN SYMPATRIC SPECIES OF THE GENUS *PHLEBOTOMUS*, WITH SPECIAL REFERENCE TO *PHLEBOTOMUS PERNICIOSUS* AND *PHLEBOTOMUS LONGICUSPIS* (DIPTERA, PHLEBOTOMIDAE)

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Summary :

The Random Amplified Polymorphic DNA assay was used to study genetic variation within and between five *Phlebotomus* species belonging to three subgenera: *P. (Larrousius) ariasi*, *P. (L.) longicuspis*, *P. (L.) perniciosus*, *P. (Paraphlebotomus) sergenti* and *P. (Phlebotomus) papatasi* sympatric in southern Spain and proven vector of leishmaniasis. Two cluster analysis were proposed: one according to sandfly species and populations, the second according individual specimens of *Phlebotomus perniciosus*, *Phlebotomus longicuspis* s.l. and intermediate morphological specimens between these species. The results obtained are closely correlated with the taxonomy classically accepted for the subgenera and with the automatic classifications made by other authors which use morphological and isoenzymatic data. The validity of the species *Phlebotomus longicuspis* is also discussed.

KEY WORDS : Phlebotomidae, sandfly, *Phlebotomus*, sympatric species, genetic polymorphism, Random Amplified Polymorphic DNA (RAPD).

Résumé : POLYMORPHISME GÉNÉTIQUE D'ESPÈCES SYMPATRIQUES DE *PHLEBOTOMUS*, AVEC UNE ÉTUDE PARTICULIÈRE PORTANT SUR *P. PERNICIOSUS* ET *P. LONGICUSPIS* (DIPTERA, PHLEBOTOMIDAE)

Le polymorphisme d'amplification de l'ADN par amorces aléatoires (RAPD) a été utilisé pour étudier la variabilité génétique inter et intraspécifique de cinq espèces de *Phlebotomus* appartenant à trois sous-genres : *P. (Larrousius) ariasi*, *P. (L.) longicuspis*, *P. (L.) perniciosus*, *P. (Paraphlebotomus) sergenti* et *P. (Phlebotomus) papatasi*. Ces espèces sont sympatriques dans le sud de l'Espagne et sont des vecteurs reconnus de leishmanioses. Deux classifications automatiques sont proposées : la première compare des populations des cinq espèces et la seconde, des individus de *P. perniciosus*, *P. longicuspis* s.l. ainsi que des spécimens présentant des caractères morphologiques intermédiaires entre ces deux espèces. Les résultats montrent une corrélation étroite avec la taxonomie classiquement admise pour les sous-genres et avec les classifications automatiques réalisées par d'autres auteurs sur les caractères morphologiques et isoenzymatiques. La validité du statut spécifique de *P. longicuspis* est également discutée.

MOTS CLÉS : Phlebotomidae, phlébotome, *Phlebotomus*, espèce sympatrique, polymorphisme génétique, RAPD.

INTRODUCTION

Sandflies (Diptera, Nematocera, Phlebotomidae) play an important role in human diseases mainly as vectors of leishmaniasis and several viroses. Their identification and classification is usually based on morphological criteria (Artemiev & Neronov 1984; Lewis 1982). From the turn of the century, the species *Phlebotomus (Larrousius) perniciosus*, *P. (L.) ariasi*, *P. (Paraphlebotomus) sergenti*, *P. (Phlebotomus) papatasi*, and *Sergentomyia minuta* have been found in Spain, and it is easy to differentiate among them. However, in recent years the species *P. (Pa.) alexan-*

dri, *P. (Pa.) chabaudi*, *P. (Pa.) riouxi*, *P. (L.) longicuspis*, *P. (L.) langeroni* and *P. (Transphlebotomus) mascittii* have also been recorded although in limited numbers. The differentiation between some of them is sometimes very difficult; for example, after the description of *P. riouxi* (Depaquit *et al.*, 1998) there is doubt if the *P. chabaudi* cited in Spain are certainly *P. chabaudi* or *P. riouxi*. Similar doubt can be raised about the species *P. longicuspis* and *P. perniciosus*, and to contribute to solve this doubt is one of the objectives of the present paper.

P. longicuspis was described in 1930 by Nitzulescu as a variety of *P. langeroni* in northern Africa and has always been considered to be very similar to *P. perniciosus*. The males of *P. perniciosus* and *P. longicuspis* have always been distinguished by the different morphology of their aedeagus which culminate in a single point in *P. longicuspis* and are bifurcate in *P. perniciosus*. For some time, the female specimens were only distinguished by biometric data (Parrot, 1936) until Leger *et al.* 1983 demonstrated that they could be morphologically distinguished by the shape of the recep-

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Species	Geographical region		Spain										Morocco				Total 166 ⁴
			Granada						Almería		Huelva						
			Loja		Restabal		Torvizcón		Turre		Higuera		Chefchaouene		Ouezzane		
	M	F	M	F	M	F	M	F	M	F	M	F	M	F			
<i>P. perniciosus</i>	–	–	–	–	13	9	8	4	–	–	–	–	7	4	45		
<i>P. longicuspis</i>	–	–	–	–	2 ³	–	1 ³	–	–	–	18 ¹	6	3 ²	–	30		
<i>P. ariasi</i>	2	–	–	–	1	–	1	11	13	1	–	–	–	–	29		
<i>P. sergenti</i>	5	1	18	4	2	–	–	–	–	–	1	–	–	–	31		
<i>P. papatasi</i>	1	–	–	–	4	1	6	19	–	–	–	–	–	–	31		

¹ : They are 11 LCA and 7 LCB specimens.

² : They are LCB.

³ : It is LCA.

⁴ : They are not included eight specimens male P.p./P.l. from Torvizcón (2) and Turre (6), which make 174 individual specimens.

Table I. – Individual specimens from natural populations studied by applying the RAPD-PCR technique. The species to which they belong, the sex and the locality in which they were captured are recorded. P.p. = *P. perniciosus*, P.l. = *P. longicuspis*, P.a. = *P. ariasi*, P.s. = *P. sergenti*, P.pa. = *P. papatasi*, M = male, H = female.

tacle at the base of the sperm ducts. In Spain, although *P. perniciosus* has been found since around the beginning of the century, *P. longicuspis* was only first recorded in 1982 (Morillas *et al.*, 1982) and the existence of female specimens has not yet been irrefutably demonstrated. Later, male specimens with intermediate forms between the two species were found (Morillas *et al.*, 1991) and the impossibility to differentiate the two Spanish species using biometric data was demonstrated (Collantes & Martínez Ortega, 1997). Recently, Benabdennbi *et al.* (1999) found in Morocco two populations of *P. longicuspis* that differed in the shape of the penan valves, the number of hairs on the internal surface of the coxites and the allelic frequencies of some isoenzymes. According to these authors, the morph male denominated LCB corresponds to *P. longicuspis* s. str. whereas one denominated LCA corresponds to a variant of *P. perniciosus*. In a previous study (Martín-Sánchez *et al.*, 1995), we showed that RAPD-PCR could be an interesting molecular technique for taxonomical, ecological and genetic population studies on Old World sandflies, and that the patterns obtained with the chosen nine primers were reproducible. By using several primers a high number of molecular characters can be generated that can be used to identify different taxa. The amplified products by RAPD-PCR can be classified into two groups: variable (polymorphic) or conserved (non-polymorphic) (Hadrys *et al.*, 1992). Both products can be used to establish associations between different species and populations.

The objectives of this study were as follows: 1) to determine the genetic divergence between *Phlebotomus* species vectors of leishmaniasis sympatric in southern Spain; 2) to evaluate the relationships among geographic populations within species and

3) to provide new data on the controversial taxonomic status of the species *P. perniciosus* and *P. longicuspis*.

MATERIAL AND METHODS

SANDFLY SAMPLES AND RANDOM AMPLIFICATION OF DNA FRAGMENTS

Specimens from natural populations of the species *P. perniciosus*, *P. longicuspis*, *P. ariasi*, *P. sergenti*, *P. papatasi* and some specimens with intermediate morphological characters between the species *P. perniciosus* and *P. longicuspis* (P.p./P.l.) were captured using CDC traps, manual techniques and adhesive traps. The captures were made in several locations in southern Spain (Almería, Granada and Huelva provinces) during 1994-1995. In addition to these, other specimens were captured in northern Morocco (Chefchaouene and Ouezzane) in 1995 with CDC traps.

Table I shows the size and geographical origin of natural populations of phlebotomine species studied by RAPD-PCR. Samples were first conserved in liquid nitrogenum (-196°C) until their morphological classification, after which they were kept in alcohol (70°C). Specimens captured with adhesive traps were only conserved in alcohol. The distal portion of the abdomen of each specimen was conserved for the morphological identification of the species. Classification of the two populations of *P. longicuspis* s.l. was made according to Benabdennbi *et al.*, 1999. Pooled and single Italian specimens of *P. perniciosus* and *P. papatasi* from cultured colonies and a pool of Spanish *P. perniciosus* (Turre, Almería province) were used as reference samples for RAPD-PCR.

The DNA purification technique and the amplification conditions were the same previously reported (Martín-Sánchez *et al.*, 1995) and carefully standardized in order to overcome the criticized lack of reproducibility of the RAPD-PCR (Black IV & DuTeau, 1997).

DATA ANALYSIS

Each amplified fragment was coded indicatively by its molecular weight in base pairs followed by the name of the primer used. The presence or the absence of the fragments in the individual specimens of each species was used to decide whether this was a conserved or a polymorphic fragment. The amplified fragments were coded as "0" (absent) or "1" (present) for each individual (dendrogram 2) or group of individuals (dendrogram 1), generating a matrix of binary data (matrix 1 for dendrogram 1 and matrix 2 for dendrogram 2, data not shown). The results obtained were used to perform a cluster analysis. Dendrograms were constructed using Jaccard's similarity index and clustering by average linkage. A correspondence analysis comparing *P. perniciosus* and *P. longicuspis* amplified fragments was performed using matrix 2 and STAF-ITCF, 1991.

RESULTS

GENERAL DATA

Random amplification of DNA extracted from 174 individual specimens (114 males and 60 females) of natural sandfly populations of the genus *Phlebotomus* with each of the nine primers produced a variable number of DNA fragments of different longitude. In Table II the conserved fragments in each of the species studied are recorded. There are a total of 143 fragments of conserved DNA in the species of which 11 are shared by at least two species. No differences were observed between the overall patterns obtained from males and females in each species examined: no band was constant for one sex and not for the other sex.

INTRASPECIFIC DATA

Phlebotomus (Paraphlebotomus) sergenti

A total of 31 specimens, 26 males and five females were studied. Among them, 30 were Spanish and one was of Moroccan origin (Chefchaouene). The band pattern obtained with the primers P94B and M13 showed a high degree of homogeneity. The greatest heterogeneity was shown by primer 006. No differences were observed between the populations with the exception of the fragment 463-004 which was conserved in all the populations except for the one from Loja.

Phlebotomus (Phlebotomus) papatasi

A total of 31 samples of Spanish origin were studied, and 20 Italian laboratory reared specimens were used as reference samples. The greatest individual variability was observed with primers 005 and 006 with which it was not possible to define conserved fragments on a species level. We did not detect any dif-

Species	Fragments of conserved DNA
<i>P. perniciosus</i>	* 843-036, 1150-036 * 250-P94B, 345-P94B, 465-P94B , 617-P94B , 713-P94B, 858-P94B , 1337-P94B * 783-004, 994-004 * 660-P94 , 783-P94 , 994-P94 , 1427-P94 1956-P94 * 432-005, 505-005, 579-005, 741-005 , 831-005, 965-005 , 1129-005 * 685-006 , 1018-006 * 300-78, 344-78 , 530-78, 650-78
<i>P. longicuspis</i> s.l.	* 994-P94, 1956-P94 * 530-78
<i>P. ariasi</i>	* 756-037 * 298-036, 388-036, 609-036 * 306-M13, 468-M13, 505-M13, 780-M13 * 250-P94B, 352-P94B, 477-P94B, 515-P94B , 713-P94B, 1244-P94B, 1500-P94B * 580-004 , 2399-004 * 660-P94, 970-P94 , 1375-P94 , 1583-P94 * 624-005, 699-005, 831-005 , 1345-005 * 804-006, 994-006 * 300-78, 530-78, 650-78
<i>P. sergenti</i>	* 358-037 , 381-037 , 626-037 * 168-036 , 220-036 , 362-036, 451-036 , 579-036, 997-036 , 1122-036 * 236-M13, 387-M13, 408-M13 , 456-M13 , 535-M13 , 761-M13 , 821-M13, 863-M13, 891-M13, 960-M13, 1055-M13, 1168-M13 * 265-P94B , 358-P94B , 404-P94B, 530-P94B , 755-P94B , 834-P94B, 925-P94B, 1306-P94B * 311-004, 375-004 , 415-004, 659-004 , 775-004 , 2181-004 * 255-P94 , 321-P94 , 502-P94 , 556-P94, 644-P94, 767-P94, 1168-P94, 1285-P94 * 553-005 , 609-005 , 726-005 756-005, 947-005, 983-005, 1018-005, 2181-005 * 353-006 , 1069-006 , 1145-006 , 1247-006 * 206-78, 217-78, 300-78, 456-78, 530-78, 564-78, 801-78, 850-78, 947-78, 1156-78, 1264-78
<i>P. papatasi</i>	* 351-037 , 374-037 , 713-037 * 284-036, 323-036, 337-036, 362-036, 418-036 , 579-036 , 741-036, 816-036, 1122-036 * 550-M13, 840-M13 * 370-P94B, 617-P94B, 677-P94B * 548-004 , 727-004 , 1295-004 * 332-P94, 620-P94 , 652-P94 , 1255-P94 * 330-78, 444-78, 468-78, 530-78, 714-78

Table II. – Fragments of conserved DNA in the species studied. Each fragment is denominated by its Molecular weight in base pairs followed by the name of the primer used. The most easily identifiable fragments, in general of the greatest intensity and, preferentially, not shared by other species, are marked in bold.

ferences between the different Spanish populations studied although differences were observed between Spanish and Italian populations with primers P94B and 78. For example, fragment 650-78 was conserved in the specimens of Spanish origin but not in reared Italian specimens.

Phlebotomus (Larrousius) ariasi

A total of 29 specimens were studied from three Spanish provinces (Almería, Granada and Huelva). Differences were observed between the specimens from eastern (Almería and Granada) and western Andalusian populations (Huelva) (E.A.P. and W.A.P., respectively). In some cases a fragment conserved in the W.A.P. is polymorphic (e.g. 305-004) or absent (as with 580-P94) in E.A.P., and sometimes the opposite occurs (e.g. 456-78 and 548-P94, respectively).

Phlebotomus (Larrousius) perniciosus

We used 45 individual specimens, 11 of Moroccan origin (Ouezzane) and the rest from two Spanish provinces (Almería and Granada). A pool of ten females of Spanish origin (Turre), and another pool of 11 males and 15 females of Italian reared specimens were used as reference samples.

The band pattern obtained with primers P94 and 005 shows a high degree of homogeneity between the different populations and specimens.

With primers 037, 036 and M13 a marked variability was expressed by the presence of bands with slight differences in their molecular weights. This variability was also observed between individuals captured from the same geographical area. In general, there was greater similarity between the Spanish and the Italian specimens than between the former and the Moroccan ones.

Phlebotomus (Larrousius) longicuspis s.l.

A total of 30 specimens, 24 males and six females were studied. These were identified by morphological characteristics as *P. longicuspis* s.l. according to classical criteria: males with pointed non-bifurcated aedeagus and females with a characteristic dilation of the distal portion of the sperm ducts (Parrot, 1936; Leger *et al.*, 1983).

P. longicuspis s.l. (Table I) can be divided into two morphologically distinct types, in accordance with Benabdennbi *et al.*, 1999: specimens with the penaeal valves with a curved distal portion denominated LCA, and specimens with a straight distal end denominated LCB. Of the 21 Moroccan males, 11 have the LCA morphology and ten specimens are LCB; the only three specimens from the Ouezzane locality have the LCB morphology. The three Spanish specimens are of LCA type.

P94 is the primer with the highest degree of homogeneity within *P. longicuspis* s.l. and together with 78 we obtained three specific fragments, 994-P94, 1956-P94 and 530-078. The first and second fragments are also conserved in the species *P. perniciosus* which also presents, with the same primer, another additional three conserved fragments (660-P94, 783-P94 and 1427-P94). It is not possible to distinguish between these two species since some specimens of *P. longicuspis* s.l. present the same conserved fragments as *P. perniciosus*. We obtained similar results with the other primers but with a greater variability and, for the same reason, it was not possible either to differentiate between this species and *P. perniciosus*.

In relation to the two morphological types mentioned, group LCA, including the three Spanish specimens, has nine conserved fragments: 660-P94*, 505-005*, 778-006**, 300-078*, 801-078, 617-P94B*, 713-P94B*, 1427-004** and 564-P94**. The fragments marked* are conserved in the species *P. perniciosus* and those marked** are conserved in all the populations of *P. perniciosus* studied with the exception of the Moroccan ones from Ouezzane. Finally, fragment 801-078 is variable in *P. perniciosus* from Turre and is conserved in the remaining populations of this species. The Spanish population of LCA has another five fragments in common with the species *P. perniciosus* although one should take into account that only three specimens are studied.

In the morphological type LCB which includes the six female studied, we did not detect any conserved fragment.

Specimens intermediate between *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (P.p./P.l.)

We studied eight male specimens from two Spanish provinces which presented intermediate morphological characteristics between both species, i.e. a penaeal valve characteristic of *P. perniciosus* and another typical of *P. longicuspis* s.l., or more specifically, type LCA. In general, with all the primers these specimens show an indistinguishable band pattern from that of the species *P. perniciosus*, with a few small exceptions. For example, fragment 579-005, which is conserved in *P. perniciosus*, is absent from the two specimens P.p./P.l. from Turre (Almería province). This fragment is also absent from all the Moroccan specimens of *P. longicuspis* s.l. and from one specimen of Spanish origin.

CLUSTER ANALYSIS AND CORRESPONDENCE ANALYSIS

From the band patterns obtained by RAPD, we obtained the Jaccard's similarity index and constructed two dendrograms to summarize the relationships among different groups. In dendrogram No 1 (Fig. 1) we considered the following 14 OTUs, in relation to

Fig. 1. – Cluster analysis according to species and geographical populations. Dendrogram constructed using Jaccard's similarity index and clustering by average linkage. Number of observations = 153 (DNA fragments, data not shown), number of variables = 14 (populations). 1 is *P. papatasi*, 2 is *P. sergenti*, 3 is *P. ariasi* from southeastern Spain, 4 is *P. ariasi* from southwestern Spain, 5 is *P. longicuspis* female from Chefchaouene (Morocco), 6 is *P. longicuspis* male LCB from Chefchaouene, 7 is *P. longicuspis* male LCA from Chefchaouene, 8 is *P. longicuspis* LCB from Ouezzane (Morocco), 9 is *P. perniciosus* from Ouezzane, 10 is *P. longicuspis* LCA from Spain, 11 is intermediate *P. perniciosus*/*P. longicuspis* LCA from Spain, 12 is *P. perniciosus* from Turre (Spain), 13 is *P. perniciosus* from Naples (Italy), and 14 is *P. perniciosus* from Torvizcón (Spain).

species, morphological type and geographical origin: *P. papatasi*, *P. sergenti*, *P. ariasi* from southeast Spain, *P. ariasi* from southwest Spain, Italian *P. perniciosus*, Spanish *P. perniciosus* from Turre, Spanish *P. perniciosus* from Torvizcón, Moroccan *P. perniciosus* from Ouezzane, Spanish LCA *P. longicuspis*, Moroccan LCB *P. longicuspis* from Ouezzane, Moroccan female *P. longicuspis* from Chefchaouene, Moroccan male LCB *P. longicuspis*, Moroccan male LCA *P. longicuspis* from Chefchaouene and the intermediate Spanish *P. perniciosus*/*P. longicuspis* LCA. We considered it more appropriate to study females of *P. longicuspis* s.l. independently from the LCB males of this species in order to avoid the interference of previous information which could have produced inaccurate results. The number of individuals in each group is recorded in Table I. We analysed a total of 153 fragments which are conserved for someone of the 14 OTUs (data not shown) and the dendrogram obtained (Fig. 1) revealed the following:

- the species *P. sergenti* and *P. papatasi* belonging to the subgenera *Paraphlebotomus* and *Phlebotomus*, res-

pectively, are clearly separate from the remaining group belonging to the subgenus *Larroussius* ($J = 0,980$);

- within *Larroussius*, 1) the species *P. ariasi* is clearly independent of the other members of this subgenus ($J = 0,886$). The two Spanish populations of *P. ariasi* are separated by a distance of 0.211. 2) At a distance of $J = 0,793$ two groups begin to separate, one includes female *P. longicuspis* and male LCB from Chefchaouene and the other is comprised of LCB from Ouezzane and LCA in addition to all the *P. perniciosus* populations. It should be noted that the LCB group from Ouezzane is comprised of only three individuals. The similarity between the Italian *P. perniciosus* and the Spanish specimens from Torvizcón is noteworthy, and the intermediate position of the Spanish LCA *P. longicuspis* and of P.p./P.l. with respect to *P. perniciosus* from northern Morocco and the group of Spanish and Italian *P. perniciosus* is also noteworthy.

In dendrogram No. 2 (Fig. 2) the OTUs are represented by 59 individuals belonging to *P. perniciosus* (11 from Ouezzane, 12 from Turre and two from Torvizcón), *P. longicuspis* s.l. (24 from Chefchaouene: six females, seven LCB males and 11 LCA males; three LCB from Ouezzane and one LCA from Turre) and six intermediate specimens *P. p./P. l.* LCA. A total of 64 conserved and variable fragments was considered (all the amplified fragments identified with accuracy in everyone of the 59 individuals, data not shown) to construct a binary matrix used to compute the Jaccard's similarity index. Clusters were observed containing members of the species *P. perniciosus* (No. 5, 6 and 7), the intermediate specimens P.p./P.l. (No. 8) and the specimens of LCA *P. longicuspis* (No. 4 and 9), whereas specimens of LCB *P. longicuspis* (No. 2, 3) and the females *P. longicuspis* (No. 1) form five small groups. In this case the three LCB specimens from Ouezzane (No. 3) are grouped together with the other LCB specimens from Chefchaouene (No. 1, 2) and become independent from the LCA. The specimens of LCA *P. longicuspis* (No. 4, 9) are integrated within both the Moroccan (No. 5) and the Spanish (No. 6, 7) *P. perniciosus*.

Correspondence analysis (Fig. 3) yielded similar results to ascending hierarchical classification. The plane formed by the first two axes ($I_1 = 20.7\%$, $I_2 = 13.2\%$) showed that *P. perniciosus* specimens, LCA type and intermediate P.p./P.l. males, are grouped and markedly distinct from *P. longicuspis* females and LCB males, which were characterized by a high polymorphism.

DISCUSSION

Application of the RAPD-PCR technique for the identification and genetic study of five species of the *Phlebotomus* genus sympatric in southern

Fig. 2. – Cluster analysis of individual specimens of the species *P. perniciosus*, *P. longicuspis* and specimens intermediate between the two (*P.p./P.l.*). Dendrogram constructed using Jaccard's similarity and clustering by average linkage. Number of observations = 64 (DNA fragments, data not shown), number of variables = 59 (individual specimens). 1 are specimens female *P. longicuspis* from Chefchaouene; 2 are specimens male *P. longicuspis* LCB from Chefchaouene; 3 are specimens male *P. longicuspis* LCB from Ouezzane; 4 are specimens male *P. longicuspis* LCA from Chefchaouene; 5 are specimens *P. perniciosus* from Ouezzane; 6 are specimens *P. perniciosus* from Turre; 7 are specimens *P. perniciosus* from Torvizcón; 8 are specimens intermediate *P.p./P.l.*; 9 is a specimen *P. longicuspis* from Spain.

Spain detected a high individual variability with, generally, numerous fragments with only slight differences in their molecular weights. Black IV *et al.*, 1993, suggested that the RAPD-PCR technique mainly amplifies highly variable regions of the genome which explains the large variability detected in a study on several aphid species. RAPD-PCR is biased in its amplification of repetitive regions (centromeric, telomeric, heterochromatic genomic regions and various classes of dispersed repetitive and mobile elements), but amplifies many unique regions as well (Black IV & DuTeau, 1997; Williams *et al.*, 1990). Polymorphisms at regions amplified by RAPD-PCR are typically manifest as the presence or absence of a band among individuals and most amplified RAPD bands are inherited in a Mendelian fashion as dominant alleles (Black IV & DuTeau, 1997).

The results of our numerical classification closely correlated with the classically accepted subgenus classification (Lewis, 1982) and with the automatic classifications made by other authors which use morphological (Rispaill & Leger, 1991) and isoenzymatic data (Pesson *et al.*, 1991). Not all the species studied showed the same degree of genetic polymorphism. The most heterogeneous was *P. longicuspis* s.l., and this was still the case after the morphological types LCA and LCB had been distinguished. In contrast, the most homogeneous species was *P. sergenti* with 67 conserved fragments.

Within the species *P. papatasi*, in spite of the marked individual variability after amplification with some primers, we did not detect genetic differences between the three Spanish populations studied. However, we did observe a degree of variability between these and

Fig. 3. — Correspondence analysis of *P. perniciosus*/*P. longicuspis*. Plane formed by the first two axes. Visible points 1, 2, 3, 4, 5, 6, 7, 8, 9, 15 (hidden point: 17), 16, 27, 30, 31 and 35 represent males type LCB and females of *P. longicuspis*. Visible points 10 (hidden points: 12, 18, 19, 20, 25, 26, 29, 34, 41, 42, 43, 51, 54, and 56), 11, 13, 14 (hidden points: 21 and 22), 23 (hidden points: 24, 28, 32, 37 and 53), 36 (hidden points: 39, 40, 44, 45, 46, 47, 48, 50, 52, 55, 57 and 59), 38 (hidden point: 49) and 58 represent *P. perniciosus*, intermediate P.p./P.l. and LCA type.

the Italian reared population which could be due to genetic selection by the culture process. Morrison *et al.* (1995) obtained very similar results to ours with isoenzyme electrophoresis.

Larrousius is a clearly separate subgenus and the genetic divergence of the species *P. ariasi* from the rest of the *Larrousius* is also clearly reflected. The differences we observed between specimens of this species from Huelva (W.A.P.) and those captured in the provinces of Almería and Granada (E.A.P.) suggest the existence of two populations resulting from genetic divergence that can be at least partially attributed to the climatic differences between both regions.

The study of *P. perniciosus* and *P. longicuspis* is especially interesting since both are vectors of *Leishmania infantum*, previously considered to be the same species (Collantes & Martínez Ortega, 1997), and because *P. longicuspis* has been observed in two completely different populations (LCA and LCB). The results obtained by RAPD-PCR show that both hypotheses can be valid: 1) *P. longicuspis* from Spain, the morphologically intermediate specimens P.p./P.l. and the LCA population of *P. longicuspis* specimens from Morocco are grouped together with the specimens of *P. perniciosus* and can, therefore, be considered as synonyms (*P. perniciosus* species); 2) The females and the LCB males of *P. longicuspis* from Morocco are separated from these and are, therefore, considered as *P. longicuspis* s.str.; the factorial analysis (Fig. 3) reinforce this hypothesis. Nevertheless, it is noteworthy that in dendrogram 1 the LCB *P. longicuspis* specimens from Ouezzane are grouped together with LCA *P. longicuspis* from Chefchaouene instead of with the LCB specimens from the

same location. This is an apparent contradiction difficult to irrefutably explain but that could be due to the very few LCB specimens from Ouezzane studied (number = 3) and the high degree of polymorphism of this species. In any case, this apparent contradiction disappears when the individual specimens are considered (dendrogram 2). In this dendrogram 2 appear five marked groups in *P. longicuspis* females and LCB males, which indicate great heterogeneity and some groups seem to be more related to *P. perniciosus* group; this heterogeneity is confirmed by correspondence analysis.

Mitochondrial introgression between *P. perniciosus* and *P. longicuspis* was inferred by Essegir *et al.*, 1999, based on mitochondrial and EF- α gene phylogenies of *Larrousius*. Also Ready & Pesson, 1999 (personal communication) provide further evidence for introgression between wild populations of these two species and for the occurrence of cryptic species. Hybridization is not uncommon among related insect species and it is known that genetic introgression is great enough to prevent the separation into two distinct lineages of the two most important vectors of malaria, *Anopheles gambiae* s.str. and *An. arabiensis* (Besansky *et al.*, 1994). The heterogeneity of the *P. longicuspis* s.str. group could reflect the occurrence of these features.

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