Sandflies of the *Phlebotomus perniciosus* complex: mitochondrial introgression and a new sibling species of *P. longicuspis* in the Moroccan Rif

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**Abstract.** The bloodsucking adult females of *Phlebotomus perniciosus* Newstead and *P. longicuspis* Nitzulescu (Diptera: Psychodidae) are important vectors of the protozoan *Leishmania infantum* Nicolle (Kinetoplastida: Trypanosomatidae) in western Mediterranean countries. The species status of the two phlebotomine sandflies was assessed, along with the epidemiological implications. Individual sandflies from three Moroccan Rif populations were characterized morphologically, isoenzymatically (by the isoelectrofocusing of alleles at the polymorphic enzyme loci of HK, GPI and PGM), and by comparative DNA sequence analysis of a fragment of mitochondrial *Cytochrome b* (mtDNA). By reference to the character profiles of specimens from other locations, including southern Spain and the type-locality countries, the Moroccan flies were placed in three lineages: first, the lineage of *P. perniciosus*, which contained two mtDNA sublineages, one (pnt) widely distributed and associated with the morphology of the male types from Malta, and the other (pna) associated with a *P. longicuspis*-like male morphology; second, the lineage of *P. longicuspis sensu stricto*, including typical forms from Tunisia; and third, a new sibling species of *P. longicuspis*. The mtDNA sublineage (pnt) of typical *P. perniciosus* was also found in some *P. longicuspis* from Morocco, indicating interspecific hybridization. The typical race of *P. perniciosus* occurs in Italy as well as in Malta, Tunisia and Morocco. It is replaced in southern Spain by the Iberian race (with the pni mtDNA sublineage). The discovery of interspecific gene introgression and a new sibling species mean that previous records of the two morphospecies do not necessarily reflect their true vectorial roles or geographical and ecological distributions.

**Key words.** *Phlebotomus longicuspis*, morphology, *Phlebotomus perniciosus* complex, sibling species, isoenzymes, mitochondrial introgression, Morocco.

**Introduction**

In this report we assess the species status of various morphological forms and populations of two closely related taxa in the *Phlebotomus perniciosus* species complex (Esseghir *et al.*, 2000) and discuss the resulting epidemiological implications.
Phlebotomus perniciosus Newstead and P. longicuspis Nitzulescu are classified in the subgenus Larroussius Nitzulescu (Seccombe et al., 1993) and, in the western Mediterranean, their haematophagous females are vectors of zoonotic leishmaniasis caused by Leishmania infantum Nicolle (Protozoa, Trypanosomatidae). The domestic dog and wild foxes are the reservoir hosts, and cutaneous and visceral human disease is frequent in rural communities, both in south-west Europe and in the Maghreb region of north-west Africa (Killick-Kendrick, 1990; Rioux & Lanotte, 1990).

Described from Malta (Newstead, 1911), P. perniciosus is widely distributed in the western Mediterranean Basin: from Portugal to Croatia in Europe, and from Morocco to Libya in north Africa, where it is found predominantly in the sub-humid and semi-arid bioclimate zones (Rioux et al., 1984). Phlebotomus longicuspis was described from Tunisia (Nitzulescu, 1930), and until 1982 was recorded only from Libya and other countries of the Maghreb (Algeria and Morocco), where it is most frequent in the semi-arid, arid and per-arid bioclimate zones (Rioux et al., 1984). Then males were reported from southern Spain, in the regions of Andalucia (Morillas Márquez et al., 1982) and Murcia (Martínez Ortega et al., 1982).

For many years considered indistinguishable, the females of P. perniciosus and P. longicuspis were separated by Léger et al. (1983) according to the form and position of the dilatation on each individual spermathecal duct: spherical and basal or heart-shaped and subbasal, respectively. As for the males, they were originally separated by the terminal structure of the aedeagi: bifurcated in P. perniciosus (Fig. 1a); simple and tapering in P. longicuspis (Fig. 1b). However, this character is subject to much individual variation (Parrot, 1936b; Morillas Márquez et al., 1991) and the identity of Spanish P. longicuspis has been questioned (Morillas Márquez et al., 1991; Collantes & Martínez Ortega, 1997).

In the course of a sandfly survey of the Rif mountains of north Morocco in 1995 (Benabdennbi & Pesson, 1998), both species were caught in the same traps set in Loubar, just outside the town of Chefchaouene (Fig. 2). Although all the females could be identified as either P. perniciosus or P. longicuspis by the morphology of their spermathecal ducts, all the males were at first identified as P. longicuspis. A careful examination of the aedeagi and the finding of a new character, the number of semi-deciduous setae on the inner face of the coxite (Benabdennbi, 1998; Benabdennbi et al., 1999), permitted the recognition of two distinctive male morphs: typical P. longicuspis (LC, formerly LC B), with each aedeagus having a straight, tapering tip (Fig. 1b), and the coxite bearing 15–29 setae; and P. longicuspis-like (PNA, formerly LC A), with an incurved aedeagal tip (Fig. 1c) and 10–16 coxite setae. An isoenzyme analysis revealed scoreable polymorphic alleles at three loci (hexokinase, glucosephosphate isomerase and phosphoglucomutase) and unambiguously grouped the PNA males with females of P. perniciosus (Benabdennbi et al., 1999). More recently a RAPD study provided supporting evidence (Martín Sánchez et al., 2000). However, the population formed by the LC males and females, with morphology
typical of *P. longicuspis*, showed a deviation from Hardy–Weinberg equilibrium in its isoenzyme genotype frequencies, which was significant in the case of phosphoglucomutase (Benabdennbi et al., 1999), and RAPD patterns remained heterogeneous (Martín-Sánchez et al., 2000).

In order to understand the reason for the genetic disequilibrium that remained within the *P. longicuspis* analysed by Benabdennbi et al. (1999), we compared individuals captured at Chefchaouene with those from two other Rif localities (Fig. 2), each characterized by a preponderance of males and females with the morphology typical of one of the two species: Taounate with 72.5% *P. longicuspis*, and Ouezzane with 76.5% *P. perniciosus*. For this, individuals were characterized in three ways: morphologically, by examination of the genitalia; enzymatically, using the three polymorphic nuclear loci that had permitted the separation of the two species at Chefchaouene; and with mitochondrial (mt) DNA haplotyping, by sequencing the 30 end of the *Cytochrome b* gene, because this marker (normally maternally inherited) had been used to relate geographical populations of many species of *Larroussius* in the Mediterranean subregion, including *P. perniciosus* and *P. longicuspis* from the countries containing the type localities (Esseghir et al., 1997, 2000; Esseghir, 1998).

With the aim of placing the results from Chefchaouene in a broader context, we also report variation in allele frequencies at the three polymorphic isoenzyme loci in southern European populations of *P. perniciosus*, and we compare newly characterized mtDNA haplotypes from Morocco and Spain with those of the distinctive Iberian sublineage previously reported from Spain and the typical sublineage found in Italy, Malta and Tunisia (Esseghir et al., 1997, 2000) (Fig. 2). These additional data help to establish the stability of the diagnostic characters for *P. perniciosus* in all countries except Morocco.

**Materials and methods**

**Sandfly collections**

Sandflies were captured overnight in modified miniature CDC light traps (Madulo-Leblond, 1983) and immediately conserved in liquid nitrogen. Most of the catches in Morocco were made 17–25 July 1995 in three localities (Fig. 2). Two are situated on the southern side of the Rif, at the boundary of the sub-humid and semi-arid bioclimatic zones: to the East, Taounate (MT samples; 500 m a.s.l.; 34°36′N,
4°40′W); and to the West, Ouezzane (MO samples; 350 m a.s.l.; 34°52′N, 5°36′W). The latter is slightly more humid, as is the third locality, Chefchaouene (MC samples; 600 m a.s.l.; 35°12′N, 5°15′W) on the Mediterranean side of the Rif. Locations and codes of additional material, captured and cryopreserved in the same conditions, are given in Fig. 2.

**Morphology**

For each individual, the genitalia were dissected and cleared in Marc-André solution (chloral hydrate/acetic acid), in readiness for morphological identification by compound microscopy (× 400–1000). The remainder of the insect was used either for mtDNA haplotyping or for isoenzyme analysis, or the head and thorax were separated for isoenzyme analysis, leaving only the abdominal remains for haplotyping.

The structure of aedeagi (Fig. 1) were photographed from males prepared as previously described (Pesson et al., 1994) and observed with a stereoscan Cambridge 100 scanning electron microscope (LEO Microscopic Electronique, France).

**Isoenzyme analysis**

Isoelectrofocusing was carried out in ultrathin agarose gels (Pharmacia® multiphor II, Amersham Biosciences, France) with the ampholyte at pH 4.0–6.5, according to the protocols described by Pesson et al. (1991). The three discriminating loci used were: 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, represented by coloured bands on the gels, were numbered chronologically for species of the subgenus Larroussius, with the more frequent allele usually having been discovered first: for HK, allele 1 = pHi 5.60, allele 2 = pHi 5.49, allele 3 = pHi 5.36, allele 4 = pHi 5.63, allele 5 = pHi 5.58, allele 6 = pHi 5.53, and 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 7 = pHi 5.69, allele 8 = pHi 5.60, and 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, and allele 8 = pHi 5.45.

The allele frequencies, tests for deviation from Hardy-Weinberg equilibrium, and UPGMA phenetic analysis based on Nei’s genetic distances were all calculated using BIOSYS-2 (Swofford & Selander, 1981). In addition, GENEPOP (Raymond & Rousset, 1995) was used to test for genotypic linkage disequilibrium and estimate F$_{ST}$ for each pair of samples.

**PCR amplification, sequencing and analysis of mtDNA**

The protocols of Esseghir et al. (1997) were followed: to extract genomic DNA; to amplify by the polymerase chain reaction (PCR) the 3′ end of the *Cytochrome b* gene (Cyt b) using primers CB3-PDR and N1N-PDR; to purify the PCR product by agarose-gel fractionation and binding to glass milk (Geneclean Kit, BIO 101 Inc, U.S.A.); to sequence the amplified fragment using each of the PCR primers and the ABI PRISM® BigDye Cycle Sequencing Ready Reaction kit (PE Applied Biosystems = ABI, U.S.A.); and, to record the DNA sequences using the ABI 373 or 377 systems and protocols. DNA sequences were aligned using SeqEd version 1.0.3 software (ABI) or Sequencher (Gene Codes Corp., U.S.A.). Phylogenetic analyses were performed with PAUP® (Swofford, 2002).

**Results**

**Morphology**

According to the aforementioned criteria, fully described by Benabdennbi et al. (1999), the males characterized from all countries (Spain, Italy, Malta, Tunisia and Morocco) could be assigned to three morphotypes: typical *P. perniciosus* (PN; Fig. 1a) in all countries; typical *P. longicuspis* (LC; Fig. 1b) only in Tunisia and Morocco; and *P. longicuspis*-like *P. perniciosus* (PNA; Fig. 1c) only in northern Morocco. There were two female morphotypes: typical *P. perniciosus* (PN) in all countries; and typical *P. longicuspis* (LC) only in Tunisia and Morocco.

**Isoenzyme analysis**

Allele frequencies at the three polymorphic loci are reported for nine populations with the PN/PNA morphology of *P. perniciosus* and for three populations with the LC morphology of *P. longicuspis* (Table 1). Only some of the isoenzyme results for the Chefchaouene (MC) flies were published previously (Benabdennbi et al., 1999). All *P. perniciosus* populations, including the PNA morphotypes, were in Hardy–Weinberg (HW) equilibrium, and there was no significant linkage disequilibrium except for two populations: in Ouezzane, Morocco (MO; HK-GPI, *P* = 0.010) and in Rio Tinto, Spain (EH; GPI-PGM, *P* = 0.046). In contrast all three populations of *P. longicuspis* from Morocco were not in HW equilibrium at the PGM locus.

Nei’s genetic distances (D) and F$_{ST}$ values were calculated among all populations (Table 2). Nei’s D-values suggest two groups of *P. perniciosus* populations, as presented graphically in the UPGMA tree (Fig. 3): (1) Italy, Malta and Morocco, where Chefchaouene (MC) is distinctive; and (2) Iberia. The F$_{ST}$ results indicate the same geographical structure and confirm the low gene flow observed between Moroccan and Iberian populations (Pesson et al., 1998).

The UPGMA tree also illustrates the genetic convergence of Moroccan *P. longicuspis* with the Iberian populations of *P. perniciosus*, although it is necessary to caution that *P. longicuspis* in Morocco does not have the genetic characteristics of a single, reproductively isolated species, as
manifested by the HW disequilibrium at the PGM locus and other indicators reported below.

**mtDNA haplotypes and lineages**

The last 279 nucleotides of *Cytochrome b* (*Cyt b*) were sequenced for 91 individual sandflies from Morocco, a single sandfly from Tunisia and 35 individual sandflies from Spain, and the sequences were aligned with those already obtained from other countries (Esseghir et al., 1997, 2000). Unique sequences (or haplotypes) were identified (Table 3) by inspection of the input data matrix and the distance matrix given by *PAUP* (Swofford, 2002). The haplotypes fell into three distinct primary lineages (*pern*, *lcus* and *lcx*), following a phylogenetic analysis based on genetic distances (Fig. 4). Haplotypes were coded to denote their lineage and the morphospecies in which they were usually found: *pern* for *P. perniciosus*, *lcus* for *P. longicuspis*, and *lcx* for a putative sibling species of *P. longicuspis*. Pairs of haplotypes differed only by 1–8 nucleotides (0.4–2.9%) within each of the three primary lineages, whereas the range of pairwise differences (and absolute genetic distances) between these lineages was 12–18 nucleotides (4.3–6.5%). In Table 4, the distributions of the newly characterized *Cyt b* sequences from Morocco are related to morphotype, sex and *Cyt b* lineage.

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Table 2. Nei’s genetic distances (above diagonal) and FST values (below diagonal) calculated from the isoenzyme data at the three polymorphic loci for nine populations of *P. perniciosus* and three populations of *P. longicuspis*

<table>
<thead>
<tr>
<th></th>
<th>Phlebotomus perniciosus</th>
<th>Phlebotomus longicuspis</th>
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<tbody>
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<td></td>
<td>EH</td>
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<td>0.0551</td>
</tr>
<tr>
<td>MC</td>
<td>0.1752</td>
<td>0.1982</td>
</tr>
</tbody>
</table>

*Key to populations is given in Fig. 2.
*PNA* is the only male morphotype recorded from this location.

sublineages, two of which were found in the Moroccan Rif (Table 3; Fig. 4). Most *P. perniciosus* from the Rif had the typical morphology (PN) and the *Cyt b* haplotype pern01, which forms with haplotype pern02 the typical sublineage (ptn) previously isolated only from Italy, Malta and Tunisia. The typical sublineage of *P. perniciosus* had been distinguished from the Iberian sublineage (pni) by two polymorphisms, at nucleotide positions 147 and 246 (diagnostic) (Esseghir et al., 1997, 2000; Table 3). The fixation of these polymorphisms in the Iberian sublineage was confirmed by characterizing the *Cyt b* sequences of 37 *P. perniciosus* from four localities in southern Spain (EH, EG, EA and EM in Fig. 2). Thirty-five flies contained haplotype pern04 and two flies contained haplotype pern05, which together form the Iberian sublineage in Fig. 4. No haplotypes of the Iberian sublineage were found in the Rif.

Haplotype pern01 of the typical sublineage was associated in the Rif not only with the typical male and female PN morphotypes of *P. perniciosus* (in Ouezzane and Taouate) but also with the typical male and female LC morphotypes of *P. longicuspis* (in Chefchaouene) (Table 4). This is consistent with interspecific hybridization and mtDNA introgression from *P. perniciosus* into *P. longicuspis*.

Eleven males of *P. perniciosus* from Chefchaouene had the PNA morphology and were characterized for *Cyt b*: all had the haplotype pern06, as did two females of *P. perniciosus* from Chefchaouene. One typical male (PN) of *P. perniciosus* from Ouezzane had a similar haplotype, pern07. The haplotypes pern06 and pern07 are considered to form the previously unreported pna sublineage (Fig. 4), which is distinguished from the other two sublineages of *P. perniciosus* by three polymorphisms, at nucleotide positions 147, 183 (diagnostic) and 246 (Table 3). Haplotypes of the pna sublineage were the only ones found in males with the PNA genitalia, all from Chefchaouene, and they were not found in any *P. longicuspis sensu lato*. However, the pna sublineage is not diagnostic for males of *P. perniciosus* with the PNA morphotype, because it was also found in two typical male PN morphotypes: one from Ouezzane, Morocco, and the second from Tunisia (Table 3).

The second of the three primary *Cyt b* lineages, lcx, has six diagnostic, fixed polymorphisms (at nucleotide positions 6, 9, 27, 54, 171 and 252; Table 3), and it had previously been isolated only from Tunisian *P. longicuspis* (Esseghir et al., 2000). It was also found only in typical male and female LC morphotypes of *P. longicuspis* from the three Moroccan localities (Table 4).

The third primary lineage, lcx, is reported here for the first time, and is characterized by three fixed polymorphisms (at nucleotide positions 27, 123 and 171; Table 3). It was found in the three Moroccan localities and, being associated only with typical male and female LC morphotypes of *P. longicuspis*, it is a marker for a sibling species.

**Combined isoenzyme profiles and mtDNA in Moroccan sandflies**

Forty-nine individual sandflies originating from Ouezzane, Taouate and Chefchaouene were fully characterized, morphologically, isoenzymically (at the HK, GPI and PGM loci) and by mtDNA sequencing. Allele frequencies were analysed at each isoenzyme locus for five populations defined by their unique combinations of morphology and mtDNA lineage (Table 5).

At the HK locus, the three groups of Moroccan *P. longicuspis* in Table 5 (all LC morphotypes, but each defined by one of the three primary mtDNA lineages) were almost monomorphic for allele 1, which also showed high frequencies (0.885–1.000) in all other populations of Moroccan *P. longicuspis* (LC morphotypes) as well as in Iberian populations of *P. perniciosus* (PN morphotypes) (Table 1). However, there were discretely lower frequencies of allele 1

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In Italian, Maltese and Moroccan populations of *P. perniciosus* (PN/PNA morphotypes) (Tables 1 and 5). In contrast, populations of *P. perniciosus* from the last three countries were characterized by a high frequency of allele 2 (0.500–1.000), which was fixed in the Chefchaouene population defined by the pna mtDNA sublineage (Table 5) but absent from all *P. longicuspis* (Tables 1 and 5).

The GPI locus showed differential polymorphism among the three primary mtDNA lineages found in *P. longicuspis* in Morocco (Table 5). This locus was almost monomorphic (0.944–1.000) for allele 1 in *P. perniciosus* (PN/PNA morphotypes) from Italy, Malta and northern Morocco, but it was less frequent in populations of *P. perniciosus* from southern Spain (0.773–0.889) and in all Moroccan populations of *P. longicuspis* (0.214–0.864) (Tables 1 and 5).

The PGM locus is highly polymorphic in *Phlebotomus* (*Larroussius*) species, and in Chefchaouene it clearly differentiated flies of the lcx mtDNA lineage from all other males and females with the typical LC morphotype (Table 5). Allele 5 was found only in the lcx mtDNA lineage, in which allele 2 was almost absent. All Moroccan *P. longicuspis* populations were not in HW equilibrium when all individuals were treated as belonging to one species (Table 1), and this is consistent with the presence of a sibling species. The frequency of allele 4 was elevated only in the subpopulation of *P. longicuspis* with the lcx mtDNA lineage and in *P. perniciosus* from Chefchaouene with the pna mtDNA sublineage (Table 5). This is another characteristic distinguishing the Chefchaouene population of *P. perniciosus* from all others (Table 1).

Therefore, based on the PGM locus, mtDNA introgression is most likely to have occurred by hybridization between *P. perniciosus* and *P. longicuspis sensu stricto*, not the sibling species. However, the hybrids do have a uniquely high frequency of allele 2 at the GPI locus (Table 5).

It is noteworthy that there are no fixed alleles diagnostic for either *P. perniciosus* or *P. longicuspis* from Morocco, even after removing the sibling species (LC morphotypes, lcx mtDNA lineage), the *P. perniciosus* variant (PNA/PN morphotypes, pna mtDNA sublineage) and the putative hybrids (LC morphotypes, pern01 haplotype of the pnt mtDNA sublineage). When present, however, allele 2 of HK was diagnostic for *P. perniciosus* and allele 2 of GPI was diagnostic for *P. longicuspis sensu lato* (Table 5).

**Discussion**

**Three species of the *P. perniciosus* complex in the Moroccan Rif**

The current findings indicate the presence of three phylogenetic species in the Moroccan Rif. One species is *P. perniciosus*, which was characterized by a distinctive isoenzyme profile and a discrete Cyt b mtDNA lineage (composed of two sublineages, typical pnt, and pna). This species includes not only the typical male and female morphotypes (PN) of *P. perniciosus* but also a male morphotype (PNA) that can be confused with *P. longicuspis* (Benabdennbi et al., 1999). The pna mtDNA sublineage is not a diagnostic marker for a sibling species with PNA male morphotypes, because it was also found in two typical (PN) males of *P. perniciosus* from Ouezzane, Morocco, and northern Tunisia (Tables 3 and 4). However, all the PNA males in...
Table 3. Alignment of mitochondrial Cyt b haplotypes (variant characters only) related to the specimens’ origins

<table>
<thead>
<tr>
<th>Cyt b Haplotype</th>
<th>Character no. in alignment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of specimens with each haplotype in different localities&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>16</td>
<td>9</td>
<td>6</td>
<td>6</td>
<td>2</td>
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</table>

<sup>a</sup>Underlined nucleotides = synapomorphic characters diagnostic for each of the three main lineages (pern, lcus, lcx), i.e. fixed polymorphisms.<sup>b</sup>Underlined numbers = data from Esseghir et al. (1997, 2000).
Chefchaouene did have the pna mtDNA sublineage as well as a characteristic isoenzyme profile (including a high frequency of allele 4 of PGM), and so there is a suggestion that the PNA aedeagus might be associated with an imperfect barrier to panmixia and incipient speciation. Direct evidence for the absence of linkage between mtDNA lineage and male morphology was provided by finding both PN and PNA males amongst the laboratory-reared progeny of a single female from El Jebha, in the Moroccan Rif (Benabdennbi, 1998). Throughout most of its range outside Morocco, the patterns of genetic variation of P. perniciosus are consistent with the isolation of populations in two commonly proposed Pleistocene Ice-Age refugia (Avise & Walker, 1998). The isoenzyme analysis (Fig. 3) indicated an Iberian cluster of populations that is distinct from an Italy–North Africa cluster (including the type locality in Malta), and the distributions of mtDNA lineages matched this (Fig. 4; Esseghir et al., 1997, 2000).

The second phylogenetic species is P. longicuspis, which was also characterized by a distinctive isoenzyme profile.
and a discrete mtDNA lineage (lcus). Genotype frequencies were in HW equilibrium at all three isoenzyme loci if specimens with the lcx mtDNA lineage were removed from the population and treated as a third, sibling species. Thus, mtDNA characterization permitted the separation of \textit{P. longicuspis sensu stricto} from males and females that shared its known diagnostic morphological characters (LC morphotypes). Benabdennbi \textit{et al.} (1999) found a departure from HW expectations at the PGM locus for \textit{P. longicuspis} caught in Chefchaouene and therefore they suspected the presence of a subpopulation, but they had no independent marker for separating the two. The males of \textit{P. perniciosus} (PN, PNA) and \textit{P. longicuspis} (LC) were shown to have a significantly different number of coxite hairs (Benabdennbi & Pesson, 1998). We can now re-analyse this data, using an analysis of variance with Scheffé test, to show that the mean number (m) of coxite hairs in males with the mtDNA lineage lcx (m = 20.3; range: 19–22; n = 14) is significantly less than that in males of \textit{P. longicuspis sensu stricto} with the lineage lcus (m = 26.4; range: 21–31; n = 26).

The relatively large pairwise genetic distances (4.3–6.5\%) between the primary mtDNA lineage characteristic of \textit{P. perniciosus} (pern) and the two found only in \textit{P. longicuspis} (lcus, lcx) indicate a divergence of these three lineages about 2–3 million years ago (mya), based on a pairwise divergence rate of 2.3\% per million years (Esseghir \textit{et al.}, 1997, 2000). The divergence of these mtDNA lineages may have accompanied allopatric speciation. However, it should be remembered that divergent mtDNA lineages may arise before speciation, because of maternal inheritance and the absence of recombination (Avise & Walker, 1998). Therefore, the presence in \textit{P. longicuspis} of two highly divergent mtDNA lineages does not in itself provide strong support for recognizing a sibling species. The necessary, complementary support for sibling species recognition comes from the coxite hair counts and especially from the isoenzyme analysis. This strongly suggests that \textit{P. longicuspis sensu stricto} and its sibling species are not just phylogenetic species (characterized by discrete isoenzyme profiles and mtDNA lineages) but are also biological species, with a substantial reproductive barrier between them. The population genetics analysis, based on isoenzymes, also indicates that \textit{P. perniciosus} is a good biological species.

The discovery of some mtDNA introgression from \textit{P. perniciosus} to \textit{P. longicuspis} does not necessarily invalidate these conclusions. However, it should be remembered that even occasional hybridization could lead to the sharing of epidemiologically important traits, as discussed in the next section.

### Evidence for mitochondrial introgression from \textit{P. perniciosus} to \textit{P. longicuspis} and its epidemiological significance

Esseghir \textit{et al.} (2000) compared mtDNA (Cyt b) and nuclear gene (EF-\alpha) phylogenies of \textit{Larroussius} species: the two phylogenies were incongruent for the \textit{P. perniciosus} complex, and mtDNA introgression between \textit{P. perniciosus} and \textit{P. longicuspis} was suspected. The current findings provide further, compelling evidence for mtDNA introgression between wild populations of these two species. In Chefchaouene, in the Moroccan Rif, some \textit{P. longicuspis} morphotypes (LC) contained a mtDNA haplotype (pern01) of a sublineage (pnt) that is usually found only in \textit{P. perniciosus} from Morocco, Tunisia, Malta and Italy. The presence of the same pern01 haplotype in the two species indicates either that introgression is still occurring or that it has occurred within the last 125,000 years, assuming the molecular clock runs no faster than 2.3\% pairwise divergence per million years (Esseghir \textit{et al.}, 2000) and that the sequencing error is not greater than 1 in 300 nucleotides. Mitochondrial DNA is usually inherited maternally and the putative hybrids were morphologically inseparable from, and isoenzymatically most similar to, \textit{P. longicuspis}. Therefore,
we conclude that fertile progeny usually result only from matings between males of *P. longicuspis sensu stricto* and the females of *P. perniciosus* or hybrids.

A comparative sequence analysis of *Cyt b* also demonstrated mtDNA introgression between closely related species within two subgenera of *Lutzomyia* (Marcondes et al., 1997; Testa et al., 2002). Like the *P. perniciosus* complex, both of these neotropical sandfly taxa contain many vectors of *Leishmania* species, and so it is important to consider the epidemiological implications of mtDNA introgression in sandflies.

Mitochondrial introgression usually results from inter-breeding. Such interspecific hybridization is not uncommon among related insect species (Arnold, 1997), and the independent assortment of many nuclear genes is axiomatic in Mendelian genetics. Therefore, it should come as no surprise if recently diverged species, such as *P. perniciosus* and *P. longicuspis*, are shown to display reticulate evolution. In these circumstances, genotypic markers as well as phenotypic traits and morphology will not always covary, partly as a result of recent ancestry (with insufficient time for ‘lineage sorting’) and partly from continuing hybridization. This absence of character covariation is not helpful to parasitologists and applied entomologists who hope that vectorial traits can be associated with easily identifiable taxonomic species, which can then be targeted for control measures. For example, much effort was made to find diagnostic markers for the major sub-Saharan vectors of malaria (Hill & Crampton, 1994), even though sibling species of the *Anopheles gambiae* Giles complex (Culicidae) are known to hybridize both in the wild and the laboratory (Black & Lanzaro, 2001), and mtDNA introgression is great enough to prevent the separation into two distinct lineages of the most important vectors, *An. gambiae sensu stricto* and *An. arabiensis* Patton (Besansky et al., 1994).

Given the evidence for the occurrence of interspecific introgression and sibling species in the *P. perniciosus* complex, it should no longer be assumed that the frequencies of certain vectorial traits can be directly related to the proportions of the morphospecies previously recorded in different geographical regions and ecological zones of north-west Africa. For example, Rioux et al. (1984) reported that *P. longicuspis* is more abundant than *P. perniciosus* in the drier bioclimates of Morocco, but only a re-examination of slide-mounted males in various collections would indicate whether authors had confused *P. longicuspis sensu lato* (morphotype LC) with *P. perniciosus* (morphotype PNA). Even then, the identification of the male morphotype LC would still leave unresolved any ecological replacement of *P. longicuspis sensu stricto* by its sibling species. Both *P. perniciosus* and *P. longicuspis* have been found naturally infected with the same strain of *Leishmania infantum* (Esseghir et al., 2000), and now reticulate evolution makes it less certain that ecological associations or vectorial roles are going to be linked solely to one taxon.

### Table 5. Allelic frequencies at the three polymorphic isoenzyme loci for Moroccan *P. perniciosus* and *P. longicuspis* grouped according to morphotype (PN + PNA and LC, respectively), mitochondrial *Cyt b* lineage and localities

<table>
<thead>
<tr>
<th>Locus and alleles</th>
<th>Morphotype PN</th>
<th>PN + PNA</th>
<th>Morphotype LC</th>
<th>LC + PNA</th>
<th>LC</th>
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</tbody>
</table>

*HK = hexokinase, GPI = glucosephosphate isomerase, and PGM = phosphoglucomutase; *P* = probability of *χ²* value occurring by chance, when testing for deviation from Hardy–Weinberg expectations of genotype frequencies.*

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Taxonomic significance of the findings: formal recognition of the new sibling species?

Those researching leishmaniasis transmission need to be aware of what information is being communicated by different taxonomic names. Two morphospecies, each with a Linnean binomial, may be good phylogenetic species and, despite occasional hybridization, may even behave most of the time as good biological species (Arnold, 1997). In these circumstances, it is usual to maintain the taxonomic species concept, with identifications based on the morphology of type specimens (Otto & Endler, 1989), not least because few laboratories have the resources for routine genetic characterization.

Until more populations have been characterized, and gene flow between them assessed, we do not favour creating or synonymizing taxonomic names in the *P. perniciosus* complex. Certainly, there is reticulate evolution, the reliance on a few characters might well mislead, as is clear from the history of the complex. Parrot (1936a) reared in the laboratory a small number of ‘pure strains’ of *P. perniciosus* and *P. langeroni var. longicuspis* and, based on this evidence for the inheritance of distinctive male genitalia, raised the latter to full species. Soon afterwards, on this evidence for the inheritance of distinctive male genitalia, Parrot & Durand-Delacre (1947) that the morphology of the aedeagus of males collected in the Sahara (Beni Ounif-de-Figuig and Tamanrasset, Algeria) was a variant of *P. longicuspis*. This variant could be the PNA morphotype, and if so Parrot & Durand-Delacre (1947) should have identified it as *P. perniciosus*. Reticulate evolution within the *P. perniciosus* complex means that all its species are unlikely to be identified by unique morphological characters or vectorial roles.

The results from Chefchaouene, in the Rif, are important because they show that some sympatric populations of *P. perniciosus* and *P. longicuspis* have the characteristics of biological species. However, more extensive characterization of populations throughout the ranges of these two morphospecies could indicate that they would be better treated as three informal incipient species (equivalent to *P. perniciosus*, *P. longicuspis* and the sibling species), with only a single taxon, *P. perniciosus*, being formally recognized. This would be appropriate only if the results from Chefchaouene were shown to be atypical, by demonstrating ongoing and significant nuclear gene flow between many sympatric populations of the three species.

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References


