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Morphological and isoenzymatic differentiation of *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera: Psychodidae) in Southern Morocco

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ABSTRACT

Morphological and enzymatic characterization of *Phlebotomus perniciosus* and *Phlebotomus longicuspis* in Morocco is reported. Twenty-nine localities in central and southern of Morocco were sampled and compared with three localities from the Rif (northern Morocco). For morphological study, sand flies were collected by sticky-paper traps. For males, the morphology of the copulatory valves (aedeagi) was examined and the number of coxite hairs was recorded. For isoenzyme analyses, specimens were collected in CDC light traps and immediately conserved at −80 °C.

P. perniciosus samples from the south of Morocco, up to 150 km from Marrakech, showed single-pointed aedeagi curved at their apices, indistinguishable from the atypical morph of *P. perniciosus*, previously described in northern Morocco. Twelve enzyme systems were tested and the qualitative analysis of zymogram profiles revealed eight polymorphic loci (glucosephosphate isomerase (GPI), phosphoglucomutase (PGM), hexokinase (HK), fumarate hydratase (FUM), malate dehydrogenase 1 (MDH1), malate dehydrogenase 2 (MDH2), 6 phosphogluconate dehydrogenase (6PGD) and aconitase (ACO)). Enzyme loci showed fixed alleles diagnostic for *P. perniciosus* (aconitase) and *P. longicuspis* (aconitase and hexokinase).

1. Introduction

Phlebotomine sand flies (Diptera: Phlebotominae) are the only known natural vectors of Protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae).

In the western Mediterranean, *Phlebotomus* (*Larroussius*) *perniciosus* Newstead and *Phlebotomus* (*L.*) *longicuspis* Nitzulescu are the proven and suspected vectors, respectively, of zoonotic leishmaniasis caused by *Leishmania infantum* Nicolle, 1908.

In Morocco, the presence of *P. pernicicosus* has been noted since 1920 in Marrakech and in Tissa (Vialatte and Parrot, 1920). Gaud (1954) and Bailly-Choumara et al. (1971) showed that it is not a very common species in the South. It shows a distinct preference for humid to semi-arid bioclimates (Rioux et al., 1984). In pre-Saharan area, *P. perniciosus* is known to be absent and *P. longicuspis* is the only *Larroussisus* species sufficiently abundant to be suspected of transmitting visceral leishmaniasis (Dereure et al., 1986).

In Chefchaouene, northern Morocco, Benabdennbi (1998) reported that females of both species were collected and identified morphologically but none of the males exhibited the bifid copulatory valves characteristic of *P. perniciosus*. Based on the number of coxite hairs, they found two groups of male morphs: typical *P. longicuspis* and *P. longicuspis*-like.

The isoenzymatic analysis classified the *P. longicuspis*-like morphs as *P. perniciosus* (Benabdennbi et al., 1999) but a fixed diagnostic allele was only found in *P. longicuspis* populations. DNA sequence analysis of mitochondrial Cytochrome *b* (Pesson et al., 2004), confirmed this identification and indicated the presence of three phylogenetic species in the Moroccan Rif: *P. perniciosus* including typical (PN) and atypical longicuspis-like (PNA) morphs; *P. longicuspis sensu stricto* and a sibling species of *P. longicuspis* (LCx).

Recently, PNA was identified morphologically in south-western Morocco (Guernaoui et al., 2005) and in Tunisia (Ghrab et al., 2006). This atypical form has not been reported yet from Algeria (Berchi et al., 2007).

In the current study, morphological and isoenzymatic characterization give evidence of a large distribution of atypical morphs of *P. perniciosus* (PNA) in southern Morocco.

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Table 1
Numbers of male (M) and female (F) of sandflies collected in different sites by both light traps (CDC) and Sticky traps (ST)

Province	Commune	Sites	Altitude (m)	CDC	ST	*P. perniciosus*				*P. longicuspis*			
						M	Fu	Fgr	Feng	M	Fu	Fgr	Feng
Marrakech	Menara-Gueliz	Semlalia	471	–	+	–	–	–	–	18	1	–	–
	Souihla	Douar Hkakma	411	+	–	–	–	–	–	15	3	2	–
El Haouz	Touama	Douar Igmir	1088	+	+	31	23	3	–	1	1	–	–
		Touflihit	1400	–	+	1	–	–	–	–	–	–	–
	Zerkten	Had Zerkten	1360	–	+	1	–	–	–	–	–	–	–
	Rhmate	Sidi Baghdad	811	–	+	7	–	–	–	5	6	–	–
		Sidi Bouyahya	759	+	+	36	6	–	–	2	–	–	–
		Dahra	667	–	+	8	–	–	–	27	–	–	–
		Ait ouaarab	764	–	+	9	–	–	–	4	–	–	–
		Ait belabass	798	+	+	9	1	–	–	1	4	–	–
		El Hajeb	731	+	–	127	41	1	68	40	38	–	29
	Stti Fatma	Stti Fatma	1772	–	+	10	1	–	–	–	–	–	–
		Tazitount	1247	–	+	5	–	–	–	1	–	–	–
		Oulmes	1170	–	+	1	1	–	–	–	–	–	–
		Aghbalou	1250	–	+	11	1	3	–	–	–	–	–
	Ourika	Tahtdoum	904	–	+	1	–	–	–	–	–	–	–
		Lkhmis	895	–	+	2	–	–	–	–	–	–	–
		Akhlij	917	–	+	1	–	1	–	–	–	–	–
		Tnin Ourika	1000	–	+	9	–	–	–	9	1	–	–
		Amkhlij	846	+	+	24	2	–	–	9	1	–	–
Azilal		Ait Majden	705	+	–	24	10	4	1	14	12	–	–
Chichoua	Taouloukoult	Taouloukoult	1237	–	+	12	–	–	–	7	–	–	–
Ouarzazate		Arghal	1439	–	+	12	2	–	–	1	–	–	–
		Aguelmous	2123	–	+	6	–	–	–	–	–	–	–
		Ighram	1995	–	+	3	–	–	–	1	–	–	–
		Aguim	1790	–	+	4	1	–	–	1	–	–	–
		Tagouimat	1866	–	+	3	–	–	–	12	4	1	-
		Amezgan	1309	–	+	–	–	–	–	8	–	–	–
		Fedragon	1132	–	+	–	–	–	–	3	1	–	–
Total						357	89	12	69	179	72	3	29

Fu: unfed female. Fgr: gravid female. Feng: engorged female.

2. Material and methods

2.1. Sand flies collection

For morphological analysis, sand flies were collected from 29 localities in central and south of Morocco, up to 150 km from Marrakech with altitudes ranging between 400 and 2100 m. Both sticky paper (A4 blank paper, coated with castor oil) and CDC light traps were used (Table 1). All specimens were conserved in 95% ethanol.

For isoenzyme analysis, sand flies were captured in CDC light traps and immediately kept at −80 °C. Four localities, in south of Morocco, were selected because of the abundance of *P. perniciosus* and/or *P. longicuspis* species: Souihla, Touama, Ourika and Rhmate. New samples from North of Morocco were studied in parallel as references: Chefchaouene, Ouezzane and Taounate.

2.2. Mounting and identification of sand flies

All specimens preserved in 95% ethanol, were cleared in potassium hydroxide for 4 h, rinsing for two hours and passed in Marc-André solution. Then, they were dissected and mounted in Hoyer's medium. Females were identified by examining the dilatation of the distal part of spermathecal ducts (Léger et al., 1983). For males, the morphology of the copulatory valves (aedeagus) was examined and the number of coxite hairs was recorded (Benabdennbi et al., 1999).

2.3. Isoenzyme analysis

For each individual, the genitalia were dissected for morphological identification. The remainder of the insect was used for isoenzyme analysis.

Isoelectrofocusing was carried out in ultrathin agarose gels with the ampholyte at pH 4.6–5 and pH 3–10, according to the protocols described by Benabdennbi (1998). The following isoenzyme systems consistently gave clearly interpretable phenotypes: glucosephosphate isomerase (GPI, E.C.5.3.1.9), phosphoglucomutase (PGM, E.C.5.4.2.2), hexokinase (HK, E.C.2.7.1.1), fumarate hydratase (FUM, E.C.4.2.1.2), malate dehydrogenase (MDH, E.C.1.1.1.37), malic enzyme (ME, E.C.1.1.1.40), 6 phosphogluconate dehydrogenase (6PGD, E.C.1.1.1.44), glycerophosphate dehydrogenase (αGPDH, E.C.1.1.1.8), isocitrate dehydrogenase (ICD, E.C.1.1.1.42) and aconitase (ACO, E.C.4.2.1.3).

The alleles, represented by coloured bands on the gels, were numbered chronologically, with the more frequent allele usually having been discovered first (Pesson et al., 2004).

2.4. Data analysis

Statistic analysis was performed with Statview software (version 5.0). ANOVA test was used to compare the number of coxite hairs and Shapiro–Wilk test for normality. Maximum likelihood method was used for decomposition of Gaussian distribution mixture.

The allele frequencies, tests for deviation from Hardy–Weinberg equilibrium, and phenetic analysis were calculated using BIOSYS-2

Table 2
Coxite hairs count in PN, PNA and LC populations. In *P. longicuspis* populations (LC = LC1 + LC2)

Population	Number of individuals		Mean	Standard deviation	Minimum	Maximum
	North	South				
PN	30	01	11.8	2.0	8	16
PNA	24	89	12.3	1.9	7	18
LC1	10	12	19.2	1.3	15	21
LC2	25	60	24.5	2.2	22	28
LC	107		23.7	2.9	15	28

(Swofford and Selander, 1981). GENEPOP (Raymond and Rousset, 1995) was used to test for genotypic linkage disequilibrium and PHYLIP version 3.6a2 (Felsenstein, 1989) was used to calculate Bootstrap by majority-rule consensus tree.

3. Results

3.1. Morphological data

Eight hundred and ten sand flies were caught. Thirty five, 8% of females were engorged and 5.5% were gravid. All engorged and gravid females were collected by CDC light traps (Table 1).

We found two female morphotypes: typical *P. perniciosus* and typical *P. longicuspis*. All *P. perniciosus* males examined showed single tipped copulatory valves curved at their apex (= PNA). Only one male displayed the bifid copulatory valves characteristic of the species (= PN), it was captured in Rhmate (Sidi Bouyahya).

65% of *P.* (*Larroussius*) *perniciosus* Newstead and 35% of *P.* (*L.*) *longicuspis* Nitzulescu were collected. For both species, the sex-ratio was in favour of males for two types of traps (Table 1).

According to altitude, we found that *P. longicuspis* and *P. perniciosus* coexist. *P. longicuspis* was the only *Larroussius* collected in plain. It peaked between 600 and 700 m. *P. perniciosus* peaked between 800 and 1000 m and persisted up to 2000 m.

In north of Morocco, we found the same results as Benabdennbi et al. (1999). The typical male specimens of *P. perniciosus* were mostly found in Ouezzane, *P. longicuspis* in Taounate and in Chefchaouene, the atypical form of *P. perniciosus* was dominant.

The number of coxite hairs was recorded for these specimens from all localities (Table 2).

A comparison of these counts using ANOVA test showed significant differences between samples of *P. longicuspis* and *P. perniciosus* PN ($P < 0.001$) or PNA ($P < 0.001$). No difference was noted between the two morphotypes PN and PNA. Specimens with a number of coxite hairs up to 14, are *P. perniciosus*, while, specimens with 19 coxite hairs or more are *P. longicuspis*.

For *P. perniciosus* population, distribution of the number of coxite hairs is not deviated from the normality ($P > 0.1$). This result confirms that there is no significant difference between two morphotypes PN and PNA in terms of number of coxite hairs. In contrast, for *P. longicuspis* population, the distribution of the number of coxite hairs is deviated from the normality ($P = 0.03$) and showed a bimodal distribution. Two groups were distinguished: group with number of coxite hairs less than 21 (LC1) and other one with more than 21 (LC2). The number of coxite hairs distribution for all populations follow a normal distribution (Fig. 1). The number of coxite hairs in LC1 group is significantly lower than in LC2 group ($P < 0.01$) (Table 2). According to Pesson et al. (2004), LC1 could correspond to the sibling species LCx and LC2 to *P. longicuspis sensu stricto*.

A comparison of the mean of the number of coxite hairs for PNA, LC1 and LC2 populations showed no significant difference between northern (N) and southern (S) populations, respectively: PNA, mN = 11.7; mS = 12.4; LC1, mN = 19.2; mS = 19.4; LC2, mN = 25; mS = 24.7.

3.2. Enzymatic data

Since MDH and ICD each showed two distinct loci (MDH1, MDH2, ICD1, ICD2), a total of 12 loci were analysed. Eight of them (GPI, PGM, HK, FUM, MDH1, MDH2, 6PGD and ACO) displayed polymorphic patterns. αGPDH, ICD1, ICD2 and ME were monomorphic. Sample sizes and allelic frequencies at each polymorphic locus are presented in Table 3.

The qualitative analysis of zymogram profiles revealed distinctive alleles at two loci. At the HK locus, allele 02 is present in all *P. perniciosus* populations and absent in all populations of *P. longicuspis*. Allele 01 of HK locus seemed to characterize PN morphotype, it was ten times more frequent in PN than in PNA morphs (Table 3). A total of 123 females were tested for HK locus. Except for one

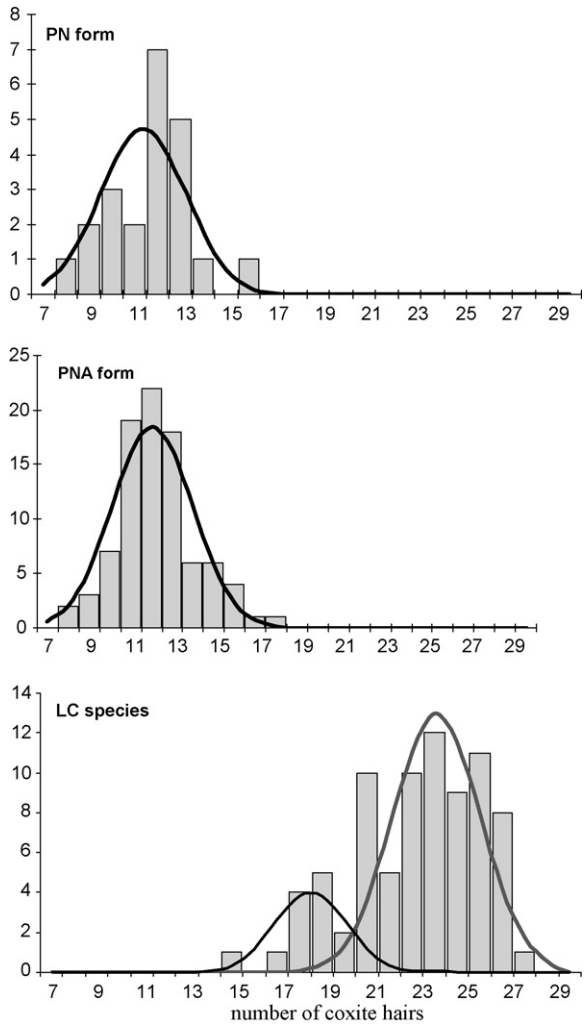

Fig. 1. Distribution of number coxite hairs in PN, PNA and LC populations.

Table 3
Allelic frequencies at eight polymorphic loci for northern and southern populations

		P. longicuspis					*P. perniciosus*				
		Taounate	Chefchaouene	Ouezzane	Souihla	Ourika	Rhmate	Touama	Taounate	Chefchaouene	Ouezzane
PGI											
(N)	pHi	18	32	11	44	31	71	31	10	22	35
1	5.30	0.833	0.656	0.955	0.943	1.000	0.852	0.839	1.000	0.977	0.929
2	5.64	0.139	0.297	0.045	0.011	0.000	0.120	0.113	0.000	0.000	0.043
3	5.00	0.000	0.031	0.000	0.034	0.000	0.014	0.016	0.000	0.023	0.014
4	6.19	0.000	0.000	0.000	0.000	0.000	0.014	0.032	0.000	0.000	0.000
5	5.09	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	5.43	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014
7	5.69	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	5.80	0.028	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000
P		1.000	0.016	1.000	1.000		0.005	0.146		1.000	1.000
PGM											
(N)	pHi	18	36	12	39	31	52	29	14	33	38
1	4.93	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	5.24	0.861	0.500	0.792	0.859	0.887	0.837	0.931	0.929	0.652	0.908
3	5.10	0.000	0.000	0.000	0.000	0.000	0.048	0.034	0.000	0.015	0.079
4	5.34	0.056	0.222	0.083	0.141	0.097	0.115	0.034	0.071	0.318	0.000
5	5.47	0.056	0.278	0.125	0.000	0.016	0.000	0.000	0.000	0.000	0.000
6	5.15	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.013
7	5.29	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.000
P		0.013	0.000	0.031	0.001	0.028	0.604	1.000	1.000	0.135	0.261
HK											
(N)	pHi	20	38	12	54	32	68	28	12	33	45
1	5.60	0.975	1.000	1.000	1.000	0.984	0.044	0.036	0.042	0.000	0.367
2	5.49	0.000	0.000	0.000	0.000	0.000	0.926	0.946	0.958	1.000	0.611
3	5.36	0.000	0.000	0.000	0.000	0.016	0.029	0.018	0.000	0.000	0.011
6	5.33	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	5.41	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011
P		1.000				1.000	0.032	1.000	1.000		1.000
FUM											
(N)	pHi	13	13	10	37	15	21	35	15	17	30
1	6.44	0.923	0.692	0.850	0.959	1.000	0.976	1.000	1.000	0.971	1.000
2	7.04	0.000	0.115	0.100	0.000	0.000	0.000	0.000	0.000	0.029	0.000
3	5.80	0.000	0.038	0.000	0.027	0.000	0.000	0.000	0.000	0.030	0.000
5	6.85	0.077	0.154	0.050	0.000	0.000	0.024	0.000	0.000	0.000	0.000
6	5.92	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000
P		0.040	0.508	1.000	1.000		1.000			1.000	
MDH1											
(N)	pHi	16	21	11	19	20	26	20	10	22	53
1	5.20	0.844	0.952	0.864	0.711	0.750	1.000	0.900	1.000	0.864	0.972
2	5.52	0.000	0.048	0.045	0.053	0.225	0.000	0.025	0.000	0.000	0.028
3	4.85	0.156	0.000	0.091	0.237	0.025	0.000	0.075	0.000	0.136	0.000
P		0.017	1.000	1.000	0.607	0.054		0.150		0.324	0.029
MDH2											
(N)	pHi	29	27	12	25	26	50	35	14	23	55
1	8.30	0.983	0.963	1.000	0.980	1.000	0.980	1.000	1.000	1.000	0.982
2	8.00	0.017	0.037	0.000	0.020	0.000	0.010	0.000	0.000	0.000	0.000
3	9.50	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.018
P		1.000	1.000		1.000		1.000				1.000
6PGD											
(N)	pHi	28	34	19	48	29	46	31	17	39	72
1	6.00	0.857	0.853	0.816	0.844	0.879	0.902	0.871	0.794	0.795	0.903
2	6.30	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.059	0.013	0.014
3	5.91	0.018	0.103	0.026	0.135	0.034	0.065	0.065	0.059	0.141	0.028
4	5.94	0.036	0.044	0.158	0.010	0.086	0.000	0.000	0.000	0.013	0.000
5	6.90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.013	0.049
6	5.88	0.018	0.000	0.000	0.010	0.000	0.000	0.032	0.000	0.013	0.007
7	6.60	0.000	0.000	0.000	0.000	0.000	0.033	0.032	0.029	0.013	0.000
8	6.45	0.071	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.000
P		0.066	0.538	0.002	0.068	0.001	1.000	0.054	0.537	0.641	0.503
ACO											
(N)	pHi	9	19	8	30	20	16	7	15	34	47
1	8.30	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.933	0.971	0.968
2	8.15	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.011
4	8.00	1.000	0.974	1.000	1.000	1.000	0.000	0.000	0.000	0.015	0.000
5	7.80	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	9.50	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000	0.011
7	8.23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011
P			1.000						1.000	1.000	1.000

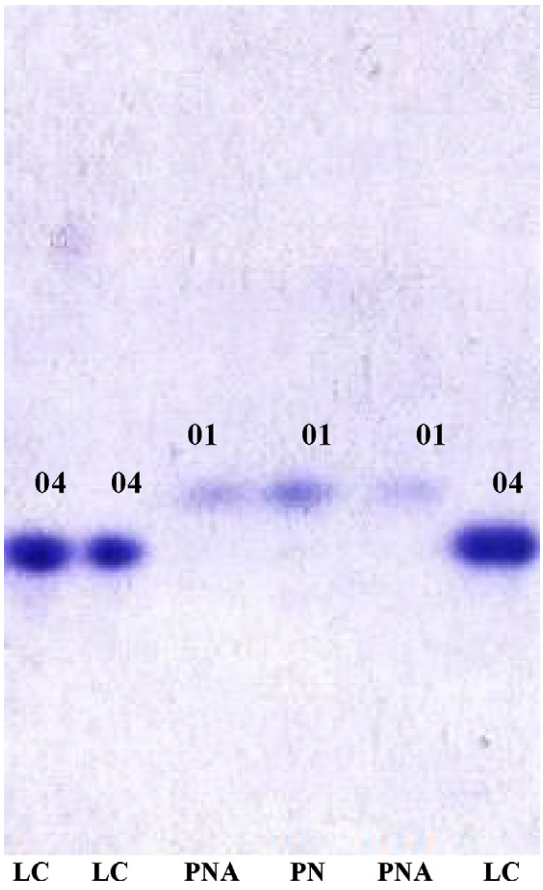

Fig. 2. IEF phenotypes of males and females PN, PNA and LC populations. Carrier ampholytes was 3-10. Allele 01 (pHi 8.30) characterizes *P. perniciosus* populations and allele 04 (pHi 8.00) characterizes *P. longicuspis* populations.

female from Taounate, all homozygotic females (0101) were from Ouezzane where the PN male morphotype is the most abundant.

At the ACO locus, except for one *P. perniciosus* female from Chefchaouene (heterozygotic 0104), allele 01 characterized *P. perniciosus* typical and atypical form and allele 04 characterized *P. longicuspis*. But this locus cannot separate between PN and PNA morphs (Fig. 2). We noted for ACO locus some genetic variability between males and females. 3/6 Alleles were present only in female specimens: one *P. longicuspis* female presented allele 05 and *P. perniciosus* females showed alleles 02 and 07. We noted the presence of allele 05 of PGM, in all *P. longicuspis* populations from north and in one individual from south (Ourika).

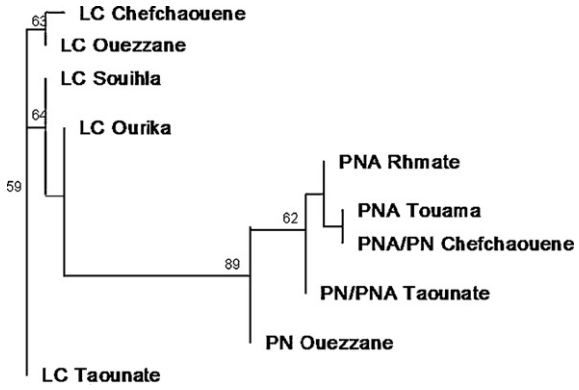

Fig. 3. Neighbour-joining tree of enzymatic data from northern and southern Moroccan populations. Bootstrap support values given by 50% majority-rule consensus tree.

Table 5
Probability of species identification according to genotypes of hexokinase (HK) and aconitase (ACO) loci

HK	ACO	N	*P. perniciosus*	*P. longicuspis*
0101	0101	3	0.66	0.33
0101	0404	63	0	1
0101	0405	1	0	1
0102	0101	8	1	0
0101	0107	1	1	0
0103	0101	1	1	0
0202	0101	56	1	0
0101	0102	1	1	0
0101	0104	1	1	0
0101	0106	1	1	0
N		136	71	65

All *P. perniciosus* populations were in Hardy–Weinberg equilibrium at the PGM locus. In contrast all populations of *P. longicuspis* were not in HW equilibrium at this locus (Table 3). Two populations from Chefchaouene presented significant linkage disequilibrium: *P. perniciosus* (MDH1–6PGD, $P = 0.04$) and *P. longicuspis* (PGI–PGM, $P = 0.003$).

Fst values and Nei's genetic distances calculated among all populations are given in Table 4. Neighbour-joining tree (Fig. 3) showed two groups: *P. perniciosus* populations and *P. longicuspis* populations. For *P. longicuspis*, the five populations are distinctive. For *P. perniciosus*, two groups were present, Chefchaouene population was associated with the two populations of southern Morocco, when, Ouezzane and Taounate were distinctive.

Table 5 summarizes the probability of species identification according to the genotype of these two loci. Specimens with allele

Table 4
Fst values (below diagonal) and Nei's genetic distances (above diagonal) calculated among all populations

	P. longicuspis					*P. perniciosus*				
	Taounate	Chefchaouene	Ouezzane	Souihla	Ourika	Rhmate	Touama	Taounate	Chefchaouene	Ouezzane
P. longicuspis										
Taounate	–	0.030	0.006	0.007	0.010	0.323	0.325	0.315	0.358	0.229
Chefchaouene	0.0692	–	0.027	0.046	0.047	0.378	0.397	0.388	0.412	0.290
Ouezzane	−0.0135	0.0516	–	0.010	0.008	0.332	0.350	0.241	0.358	0.235
Souihla	0.0130	0.1478	0.0197	–	0.007	0.335	0.333	0.321	0.351	0.237
Ourika	0.0308	0.1647	0.0091	0.0169	–	0.322	0.322	0.307	0.347	0.224
P. perniciosus										
Rhmate	0.5515	0.5572	0.5679	0.6236	0.6222	–	0.003	0.005	0.012	0.018
Touama	0.5288	0.5217	0.5517	0.5848	0.6088	−0.0005	–	0.005	0.016	0.019
Taounate	0.5789	0.5535	0.6008	0.6492	0.6840	0.0110	0.0028	–	0.014	0.019
Chefchaouene	0.5615	0.5632	0.5645	0.6521	0.6589	0.0541	0.0736	0.0545	–	0.039
Ouezzane	0.4341	0.4814	0.4709	0.5571	0.5514	0.1022	0.0943	0.0857	0.1698	–

02 of HK locus or allele 01 of ACO locus are *P. perniciosus* at 99.7% and 99%, respectively. Specimens with allele 04 of ACO locus are *P. longicuspis* at 99.4%. All specimens with genotype 0101 of HK locus and 0404 for ACO locus are *P. longicuspis* (probability = 1). All specimens with genotype 0101 for ACO locus and 0202 or 0102 for HK locus were *P. perniciosus* (probability = 1).

4. Discussion

The confusion in morphological diagnosis between *P. perniciosus* and *P. longicuspis* had been resolved in Moroccan Rif by isoenzyme (Benabdennbi et al., 1999) and mitochondrial DNA characters (Pesson et al., 2004). In 2005, Guernaoui et al. reported the presence of atypical morphs of *P. perniciosus* in south-western Morocco. Our results completed these data and gave evidence that these atypical morphs (PNA) are widespread in the south of Morocco.

Statistical analysis of coxite hairs countings, confirmed the significance of this criterion previously described by Benabdennbi et al. (1999), to separate males of both species. *P. longicuspis* displayed in the south the same bimodal distribution than in the north with some males bearing a lower number of setae.

Isoenzymatic characterization showed at the HK locus, one allele (02) present only in *P. perniciosus* populations. The frequency of this allele was elevated in the populations of PNA (0.92–1.00) compared with typical (PN) population of Ouezzane (Table 3). In addition, a new isoenzymatic marker was found: aconitase can distinguish between *P. perniciosus* and *P. longicuspis*.

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 some loci, especially at the PGM locus and as showed in the distribution of the number of coxite hairs in males. Allele 05 of PGM is not fixed but seems to be present only in the *P. longicuspis* sibling species (LCx) characterized in Chefchaouene, by Cytochrome *b* mtDNA sequence (Pesson et al., 2004). The current findings indicate that this PGM allele is also present in southern Morocco where it had been characterized in only one individual from Ourika. In subsaharian Burkina Faso, *P. longicuspis sensu stricto* was identified morphologically and by sequencing of the same fragment of Cytochrome *b* mtDNA and LCx was not recorded in this area (Depaquit et al., 2005).

According to altitude, *P. longicuspis* was sympatric with *P. perniciosus*, particularly in the mountainous area. In the plain, only *P. longicuspis* was present. This observation cannot confirm the previously described ecology of *P. longicuspis* (Rioux et al., 1984; Guernaoui et al., 2006) in Morocco as the distribution of this species should be revised with a separation between *P. longicuspis s.s.* and its sibling species.

The two morphotypes of *P. perniciosus* did not have the same repartition. PN males were collected at 96.9% in northern Morocco, while only one was found in southern Morocco where the majority of *P. perniciosus* males were PNA morphs. The dominance of atypical form of *P. perniciosus* in south of Morocco, until Ouarzazate city and the description of *P. longicuspis* "variant" similar to PNA by Parrot and Durand-Delacre (1947) in Sahara of Algeria, indicate that this form is more widespread in the south. This distribution may be linked to climatic conditions and this morph can be more adapted to environmental conditions found in this area. In north-east Spain, Aransay et al. (2004) found a significant variation in the relative abundance of *P. perniciosus* according to bioclimatic zone and altitude, so, it was predominant in the warmest and driest bioclimatic zones. In our results, all localities prospected in southern Morocco are in arid climate but humid climate appears in mountainous area where only *P. perniciosus* persists.

We concluded that in Morocco two *P. perniciosus* morphotypes are present: PN located in the north and PNA extending to the south. Hexokinase and aconitase isoenzymes are efficient markers to distinguish *P. perniciosus* and its close related species *P. longicuspis*. The latter is widely distributed and there are some morphological and isoenzymatic evidences of the presence of the sibling species, reported from Rif region, in the south. This should be confirmed on more samples and using other molecular typing.

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