

CHINESE PHLEBOTOMINE SANDFLIES OF THE SUBGENUS *ADLERIUS* NITZULESCU, 1931 (DIPTERA: PSYCHODIDAE) AND THE IDENTITY OF *P. SICHUANENSIS* LENG & YIN, 1983 II - GENOTYPING AND NUMERICAL ANALYSIS

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Summary:

The authors carried out isoenzyme characterization and genotype comparison studies for *Phlebotomus chinensis* and *Phlebotomus sichuanensis* imagoes collected from their holotype/paratype original localities: West Mountain, West Suburb, Beijing and Lixian County, Sichuan Province. Numerical analysis was carried out and the dendrogram of four species of *Adlerius* was drawn up according to morphological characteristics of these four species. The results showed that *P. chinensis* and *P. sichuanensis* can be identified enzymatically in PGM and the MDH-2 allele also differs in *P. chinensis* from Beijing and *P. sichuanensis*. The dendrogram of four *Adlerius* species showed that *P. fengi* and *P. longiductus* are very similar and they clustered first, then *P. sichuanensis* combine with them to form a combined group. *P. chinensis* is a separate group from other three. These two groups come to combine at last. Above results have proved that *P. sichuanensis* is an isolate species instead of a so-called large type *P. chinensis*.

KEY WORDS : *Phlebotomus sichuanensis*, isoenzyme characterization, genotyping, numerical analysis, taxonomy.

Résumé : LES PHLÉBOTOMES DE CHINE DU SOUS-GENRE *ADLERIUS* NITZULESCU, 1931 (DIPTERA: PSYCHODIDAE) ET IDENTITÉ DE *P. SICHUANENSIS* LENG & YIN, 1983. II – GÉNOTYPE ET ANALYSE NUMÉRIQUE

Les auteurs ont réalisé une comparaison des isoenzymes et des génotypes de *Phlebotomus chinensis* et *Phlebotomus sichuanensis* collectés dans les localités des holotypes et paratypes des montagnes de l'ouest et de la périphérie ouest de Beijing et Lixian, Province du Sichuan. Une analyse numérique et un dendrogramme de quatre espèces du sous-genre *Adlerius* ont été faites selon leurs caractéristiques morphologiques. Il résulte que *P. chinensis* et *P. sichuanensis* peuvent être identifiés au plan enzymatique pour l'étude de la PGM et également que l'allèle MDH-2 est différent chez *P. chinensis* provenant de Beijing et *P. sichuanensis*. Le dendrogramme des quatre espèces d'*Adlerius* montre que *P. fengi* et *P. longiductus* sont très proches. *P. chinensis* constitue un groupe séparé de celui des trois autres espèces. Ces résultats prouvent que *P. sichuanensis* est une espèce en soi et non un représentant de grande taille de *P. chinensis*.

MOTS CLÉS : *Phlebotomus sichuanensis*, isoenzyme, génotype, analyse numérique, taxonomie.

INTRODUCTION

Four species of *Adlerius* phlebotomine sandflies have been recorded in China, namely *Phlebotomus chinensis* Newstead, 1916; *P. fengi* Leng & Zhang, 1994; *P. longiductus* Parrot, 1928 and *P. sichuanensis* Leng & Yin, 1983. *Adlerius* phlebotomines are the main vectors of visceral leishmaniasis (VL) in China, three of them are incriminated VL vectors (Leng & Zhang, 1992; Leng & Zhang, 1994; Yin *et al.*, 1985;

Zhang & Leng, 1997) and *P. fengi* is considered a potential VL vector for the southwestern mountainous region (Leng & Zhang, 1994). Xiong *et al.* (1990) synonymized **Ps** to **Pc** and considered **Ps** to be a large type of **Pc**. In the first part of the present study (Leng & Zhang, 2001), the authors proved the isolate identity of **Ps** by taxonomical and zoogeographical studies. In this report genotyping and numerical analysis are studied.

MATERIALS AND METHODS

STUDIED SPECIMENS

Pc and **Ps** imagoes were collected from their holotype/paratype original localities: West Mountain, West Suburb, Beijing and Lixian County, Sichuan Province respectively. **Pf** specimens measured are the holotype and paratypes preserved in Jinan University (Leng & Zhang, 1994) and the measurements of **Pl** are quoted from original descriptions and well known papers (Parrot, 1928; Wang *et al.*, 1966; Artemiev, 1980; Lewis, 1982; Feng, 1983).

ABBREVIATIONS:

Pc: *Phlebotomus chinensis*, **Pf:** *Phlebotomus fengi*, **Pl:** *Phlebotomus longiductus*, **Ps:** *Phlebotomus sichuanensis*, VL: visceral leishmaniasis.

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ISOENZYME AND GENOTYPE ANALYSIS

Pc and **Ps** imagoes were collected from the places respectively mentioned above. After stunning by chloroform the sandflies were individually one by one transferred into labeled small plastic tubes for immediate preservation in liquid nitrogen at -196°C . Before electrophoresis the fly's head was removed for species identification and the rest of each fly was homogenized in 5 μl of enzyme stabilizer (2 mM EDTA, 2 mM 6-aminocaproic acid, 2 mM dithiothreitol) in the well of a plastic titration plate with a glass rod. Each homogenate was absorbed into a piece of 4×6 mm filter paper before it was inserted into one end of 12.5 % horizontal starch gel. 0.1M Tri-0.1M Maleic-0.01M EDTA buffer system was used. After electrophoresis, the gel was sliced horizontally into three to four slices 1mm in thickness. Each slice is stained for different enzymes. Detection of following enzymes was attempted: alcohol dehydrogenase (ADHE-E.C.1.1.1.1); aldehyde oxidase (AO-E.C.1.2.3.1); aspartate aminotransferase (ASAT or GOT-E.C.2.6.1.1); esterase (ES-E.C.3.1.1.1); glucose-phosphatase isomerase (PGI-E.C.5.3.1.9); glycerol-3-phosphate dehydrogenase (a-GPD-E.C. 1.1.1.8); hexokinase (HK-E.C.2.7.1.1); lactate dehydrogenase (LDH-E.C.1.1.1. 27); malate dehydrogenase (MDH-E.C.1.1.1.37); phosphoglucumutase (PGM-E.C.5.4.2.2). The staining technique used was similar to those described by Siciliano & Shaw (1980).

NUMERICAL ANALYSIS

17 selected morphology characteristics of the male (12) (A3: antenna 3; A3/L: antenna 3/labrum; A4: antenna 4; WL: wing length; AF: ascoid formula; HC: hairs on inner surface of coxite; FHC: farthest hair from base of coxite; ST: subapical tubercle of aedeagus; GF: genital filament, Style; Aed: aedeagus; F/P: genital filament/genital pump) and female (5) (A3, A3/L, A4, WL, CDS: common duct of spermatheca) adult of the four species in subgenus *Adlerius* were used as operational taxonomic units, numerical analysis was carried out. SPSS 10.0 statistic software (Liu, 2000) was used to the hierarchical cluster analysis, and the cladistic dendrogram of four species of *Adlerius* was plotted up.

RESULTS

ENZYME LOCI

Among the 10 enzymes, LDH and ADH showed no activity although treated with several kinds of buffer systems and adjusted the conditions for electrophoresis. These two enzymes may be absent in the sandflies if the technique fails to detect them. HK and ES appeared

very dimly and the diffuse bands were difficult to identify. AO displayed two bands only in the TVB buffer system. The activity of ASAT was very unstable.

MDH with two loci migrated anodically (MDH-1, MDH-2) and one locus migrated cathodically (MDH-3) (Table I, Fig. 1a). α -GPD with a single locus manifested in all phlebotomines examined among which only a few showed two bands (Table I, Fig. 1b). PGM with one locus comprised four alleles (Table I, Fig. 1c). PGI with two loci, namely PGI-1 (extremely active) and PGI-2 (migrates faster than PGI-1) (Table I, Fig. 1d).

DIFFERENT GEOGRAPHICAL POPULATION OF *P. CHINENSIS*

Allozyme phenotypes appeared almost the same except in MDH-2 which revealed slight variations (RMD) in the 80 of the alleles in *P. chinensis* from Beijing but was absent in the **Pc** from Sichuan. (Table I, Fig. 1).

P. CHINENSIS AND *P. SICHUANENSIS*

Adlerius phlebotomines were identified mainly by the male morphology of the species. Though their females resemble them morphologically but their enzymic characteristics are different. The characteristic enzyme loci of MDH-2 and PGM differ between these two sandflies. The alleles of locus in MDH-2 situated at 80 (or absent) and 84 for **Pc** and **Ps** respectively. The locus

Enzyme phenotypes*	<i>P. chinensis</i> (BJ)	<i>P. chinensis</i> (SC)	<i>P. sichuanensis</i>
MDH-1 100	54	32	30
MDH-2 84	0	0	10
80	24	0	0
α -GPD 100	32	25	13
100/103	0	0	1
97	0	0	1
95	0	1	0
PGM 100	17	12	1
108	0	0	9
100/105	5	2	0
100/108	0	0	1
103/108	0	1	1
PGI-1 100	43	40	18
100/96	5	1	3
100/94	1	0	0
PGI-2 110	6	15	8
110/114	8	4	3
114	0	0	1

* The nomenclature of the enzyme phenotypes was based on an arbitrary designation of "100" for the distance of the most common band at the locus considered in *P. chinensis*. Subsequently designation were derived from measurements relative to the standard "100".

(BJ) : specimens from Beijing; (SC): specimens from Sichuan.

Table I. – Number of allotypes at different loci in *P. chinensis* and *P. sichuanensis*.

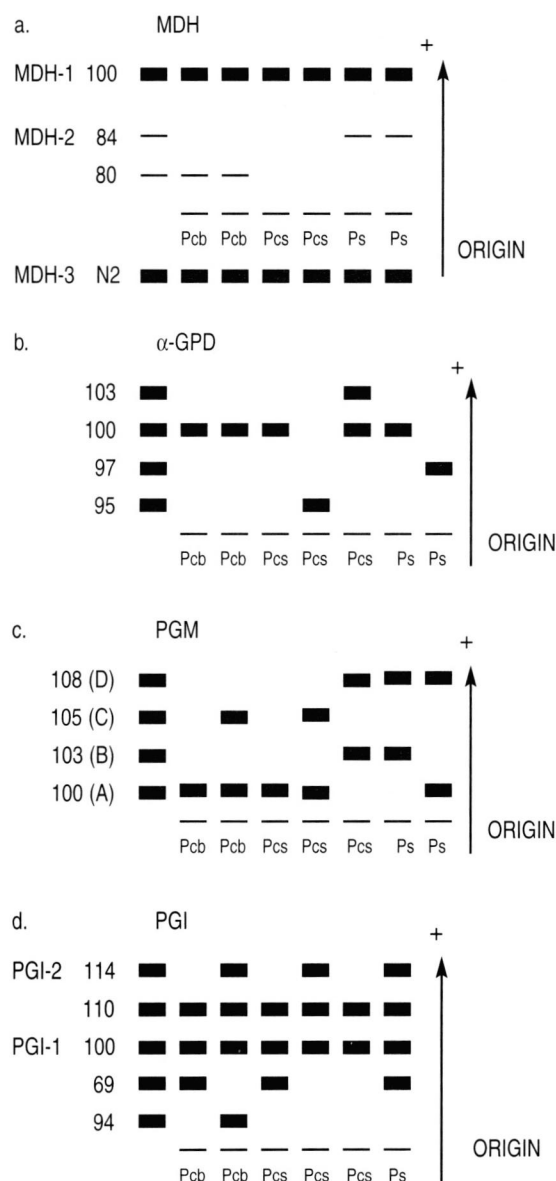


Fig. 1. – Composite diagram of enzyme phenotype in starch gel electrophoresis for *P. chinensis* and *P. sichuanensis*.

Pcb: *P. chinensis* (Beijing); Pcs: *P. chinensis* (Sichuan); Ps: *P. sichuanensis*.

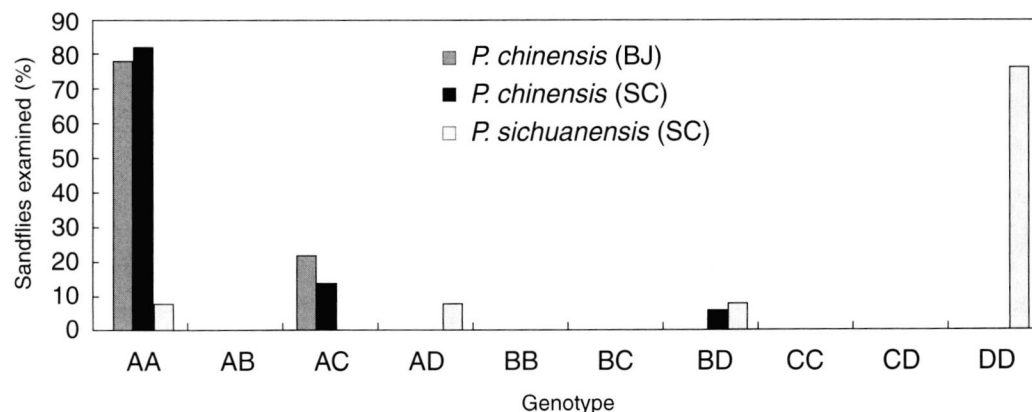


Fig. 2. – A comparison of PGM genotype of *P. chinensis* and *P. sichuanensis* from Beijing (BJ) and Sichuan (SC).

Species	GI*	GD*
<i>P. chinensis</i> (BJ) – <i>P. chinensis</i> (SC)	0.907	0.098
<i>P. chinensis</i> (BJ) – <i>P. sichuanensis</i>	0.702	0.353
<i>P. chinensis</i> (SC) – <i>P. sichuanensis</i>	0.783	0.245

(BJ) = specimens from Beijing, (SC) = specimens from Sichuan.

* $I = \sum X_i Y_i / \sqrt{\sum X_i^2 Y_i^2}$; $GI = I_{XY} / \sqrt{I_X I_Y}$; $GD = -\log_e GI$ (Nei, 1972; Zhen, 1987).

Table II. – Average genetic identity (GI) and genetic distance (GD) between *P. chinensis* and *P. sichuanensis*.

in PGM for genotype AA(100) is over 77 % and 8.3 % and genotype DD(108) is negative and 75 % in **Pc** and **Ps** respectively (Fig. 2). The GI and GD calculated (Nei, 1972; Zhen, 1987) from allelic frequencies of six loci for these two flies are listed in Table II.

NUMERICAL ANALYSIS

Rescaled distance cluster combined analysis (hierarchical cluster method: Liu, 2000) of morphology characters of four species showed that **Pf** and **Pl** are very closely related species, they cluster first, then **Ps** combine with them to form a group. **Pc** is another group. These two group cluster last. (Fig. 3).

DISCUSSION

Perrotey *et al.* (2000) separated two sympatric species in the subgenus *Adlerius*, *P. simici* and *P. brevis*, by means of agarose enzyme electrophoresis, and found differential patterns at the PGM and HK loci. Benabdennbi *et al.* (1999) separated two closely related species, *P. longicuspis* and *P. perniciosus*, by their isoenzyme patterns using isoelectrofocusing of seven enzyme systems. In this study, **Pc** and **Ps** could be identified enzymatically in PGM, their differences are also demonstrated in **Pc** Beijing strain and in **Pc** Sichuan strain living in the same area as **Ps**. The MDH-2 allele is also different in the **Pc** from Beijing

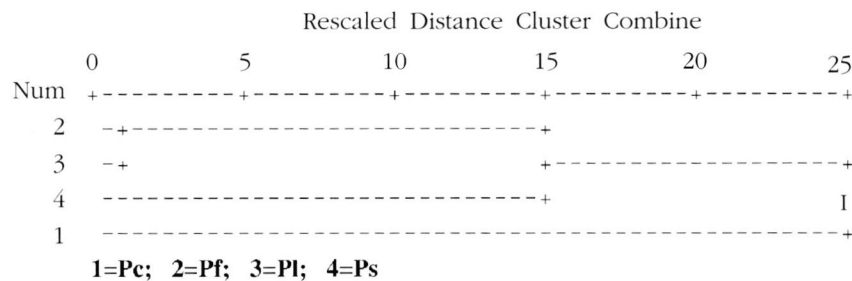


Fig. 3. – The cladistic dendrogram of numerical analysis of four species of *Adlerius*.

Cluster analysis of 17 morphology characters of four species of *Adlerius*; SPSS 10.0 statistic software.

and **Ps**, but it is absent in the **Pc** from Sichuan (Fig. 1a) (it may be a genetic variety caused by geographical separation and ecological environment factors). From the dendrogram of morphological numerical analysis of four species of *Adlerius*, it is clear that **Pf** and **Pl** are very close species and they cluster first, then **Ps** combines with them to form the first group. **Pc** is another separate group. These two groups combine last. In the present study, **Ps** specimens are collected from above 900 m altitude, Lixian County, Northwestern Sichuan where its holotype or paratypes were first collected. **Pc** specimens were collected from: 1) West Mountain, West Suburb, Beijing where its holotype was collected in 1915; 2) Nanping County, Northwestern Sichuan where **Pc** and **Ps** are parapatrically distributed. ISS have not collected specimens from the place where the original holotype or paratypes of **Pc** and **Ps** were formally collected. Their specimens were collected from the parapatric zone for **Pc** and **Ps**. Data of those specimens have not been given for size distinction but just according to naked eye to identify large and small type **Pc**. So it is hard to avoid incorrect identification and confusion with the original specimens used in their work (Qiao & Lu, 1989; Zhao *et al.*, 1997; Zhao *et al.*, 1998).

CONCLUSION

Combined with the morphological difference, geographical distribution, isoenzyme study, numerical analysis and the development of their premature stages of four species of *Adlerius*, **Ps** is an isolate species instead of a large type of **Pc**.

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