FIRST RESULTS OF THE APPLICATION OF ISOZYME ANALYSIS TO STUDY OF *CULICOIDES* (DIPTERA: CERATOPOGONIDAE)

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ABSTRACT. Results of a biochemical study on the genus *Culicoides* are reported. A method of analysis using six discriminant enzymatic systems was used and the results obtained on zymograms are detailed. A genetic study of *C. nubeculosus*, based on phosphoglucomutase was carried out. The processing of the data obtained, by means of correspondence analysis, provides an interesting comparative study between morphologically related species.

INTRODUCTION

Although techniques involving biochemistry in entomology are not new, they have rarely been used for the Ceratopogonidae. Wirth and Morris (1985), after extraction of cuticular hydrocarbons, analyzed the complex *Culicoides variipennis* by gas chromatography. For analytical purposes, we have adapted an isoenzymatic methodology to the *Culicoides* utilizing horizontal electrophoresis on a starch gel (Waller 1986). The zymograms obtained enabled us to begin a biochemical study of the genus and a genetic study of one species. The data were then processed by correspondence analysis which made it possible to compare the different species.

MATERIALS AND METHODS

Electrophoresis of the Culicoides. Harris and Hopkinson (1978) elaborated the techniques to be used; their applications in entomology, particularly with mosquitoes, were developed by Steiner and Joslyn (1979).

Culicoides used for enzymatic studies were identified after capture and CO_2 narcosis. While still alive, they were then frozen at −80°C and individually preserved. When used, they were ground in their frozen state in 10 μl of distilled water. A glass rod and a refrigerated alveolated porcelain plate were used for grinding. The extracts were then absorbed with filter paper wicks (Whatman no. 3, 9 mm long × 8 mm wide).

Two different buffers were used and tested with each enzyme: 1) TCE buffer (pH 7.4) composed of: Tris (hydroxymethyl)aminomethane (0.1 M), Citric acid monohydrate (0.028 M), Ethylenediaminetetraacetic acid disodium salt (0.01 M), and 2) TME buffer (pH 9.5) composed of: Tris(hydroxymethyl)aminomethane (0.1 M), Maleic acid (0.1 M), Ethylenediaminetetraacetic acid disodium salt (0.01 M), $MgCl_2$ (0.01 M).

These buffers were used pure in the electrode tanks and diluted to 1/9 with distilled water to prepare the 11% starch gel which was poured in a 1 cm high Plexiglas container, whose internal dimensions were 17 × 19 cm (migration plate). This plate was then wrapped in a polyethylene sheet and left overnight at room temperature. The quantity of gel used was approximately 300 ml.

The migration plate was cooled to 4°C one hour before the beginning of the migration. The gel was then perforated with a 15-tooth calibrated comb impregnated with a solution of bromophenol blue, which served as a migration indicator. The sample wicks containing the *Culicoides* extracts were inserted into the slots. The voltage was maintained at a constant level of 150 V. During the entire electrophoretic process the temperature was maintained at 4°C. The duration of migration averaged 5–6 hours based on the shifting of the color indicator (bromophenol blue).

The stain assay was prepared one hour before the end of the migration. Following electrophoresis, the gel, free of its edges, was cut into five equal slices (2 mm). Hence five thin gels were obtained, and one single migration enabled the simultaneous testing of five different enzymes (one per sliced gel). Coloration lasted 30 minutes to 3 hours, depending on the enzyme to be revealed.

Twenty-four enzyme[3] systems were tested using two staining techniques.

The first one consisted of simple immersion of the thin gel in the stain assay. The enzymes studied through this process were: Lactate dehydrogenase (Ldh) E.C. 1.1.1.27., Aspartate amino transferase (Aat) E.C. 2.6.1.1., Esterase (Est) E.C. 3.1.1.1., Hydroxybutyrate dehydrogenase (Hbdh) E.C. 1.1.1.30., Xanthine dehydrogenase (Xdh) E.C. 1.2.1.37., Xylose dehydrogenase (Xld) E.C. 1.1.1.175., Glyoxalase (Glu) E.C. 4.4.1.5., Acid phosphatase (Acp) E.C. 3.1.3.2., Glucose dehydrogenase (Gld) E.C. 1.1.1.47.

In the second technique, the gel was covered

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[3] Each enzyme is followed by an abbreviation and the international nomenclature.

with the stain assay added with 2% agarose (overlay). This method was used for: Phosphoglucomutase (Pgm) E.C. 2.7.5.1., Malic enzyme (Me) E.C. 1.1.1.40., Hexokinase (Hk) E.C. 2.7.1.1., Nucleoside phosphorylase (Np) E.C. 2.4.2.1., α-Glycerophosphate dehydrogenase (α-Gpdh) E.C. 1.1.1.8., Malate dehydrogenase (Mdh) E.C. 1.1.1.37., Adenylate kinase (Ak) E.C. 2.7.4.3., Isocitrate dehydrogenase (Idh) E.C. 1.1.1.42., Mannose phosphate isomerase (Mpi) E.C. 5.3.1.8., Aldehyde oxydase (Ao) E.C. 1.2.3.1., Phosphoglucose isomerase (Pgi) E.C. 5.3.1.9., 6 Phosphogluconate dehydrogenase (6Pgdh) E.C. 1.1.1.44., Glucose 6 phosphate dehydrogenase (G6pdh) E.C. 1.1.1.49., Octanolic dehydrogenase (Odh) E.C. 1.1.1.73., Guanine deaminase (Gda) E.C. 3.5.4.3.

Ten species of *Culicoides* of the Palearctic region were tested: *C. achrayi* Kettle and Lawson, *C. nubeculosus* Meigen, *C. chiopterus* Meigen, *C. dewulfi* Goetghebuer, *C. obsoletus* Meigen, *C. musilator* Kremer and Callot, *C. punctatus* Meigen, *C. circumscriptus* Kieffer, *C. clastrieri* Callot, Kremer and Deduit, and *C. odibilis* Austen.

Data Analysis. A binary data matrix has been elaborated, by coding the observed isoenzymatic bands obtained for the different zymograms belonging to the different species of *Culicoides* (cf. Results). Then the matrix was introduced into an IBM-PC XT (512 ko memory), and a study was performed by means of a program[4] enabling us to process the matrix by factorial correspondence analysis (CFA) (Benzecri 1982).

RESULTS

Isozymes. Among the 24 enzymatic systems tested with the two buffers, only eight (Pgi, Hk and Ao in TCE buffer and Pgm, Me, Idh, Ak and α-Gpdh in TME buffer) showed reproducible patterns. Two of them (Ak and α-Gpdh) are monomorphic whatever the species of *Culicoides* tested, and the six others are polymorphic. Our interpretation is based on the results obtained on these six ones.

The 10 above-named species of *Culicoides* were tested using the discriminant enzymatic systems. Two types of isoenzymes were found: 1) enzymes with only one band (Me, Pgi and Ao) but located on different levels, 2) enzymes in which one or two bands were detected, depending on the specimens (Pgm, Hk and Idh).

The simplest hypothesis regarding the enzymatic systems presenting either one or two bands is to assume that they are monomeric. Therefore, a single-band zymogram corresponds to a homozygote and a two-band zymogram to a heterozygote individual. This assumption could only be confirmed by a genetic analysis similar to the one carried out for Pgm with *C. nubeculosus* (see phosphoglucomutase genetic study). Taking into account the aforementioned reservations, we shall consider such enzymes as monomeric, the bands are numbered according to increasing mobility. A drawing of the enzymatic bands obtained for each species was traced from each zymogram. This comparative study of isozymes leads to the elaboration of "juxtaposed zymograms" which distinguish each species biochemically. All the band phenotypes which were detected throughout our experiments are presented on these zymograms (Figs. 1 and 2).

Phosphoglucomutase Genetic Study. Among the enzymatic systems tested, phosphoglucomutase (Pgm) is one of the more easily detectable. This enzyme was used to distinguish different insect populations such as *Drosophila* spp. (Ayala 1982) or mosquitoes (Posey and Brown 1977). We used this enzyme to demonstrate the allelic diversity of the Pgm locus of the species reared in our laboratory, *C. nubeculosus* (Waller et al. 1986). The screening of 131 individuals from this strain enabled us to calculate the genotypic and allele frequencies, and to verify if the population is at a Hardy-Weinberg equilibrium for the Pgm locus.

From a given band phenotype, called the zymogram, the genetic analysis enabled us to define the type of enzyme (monomeric, dimeric, polymeric) as well as the number of loci involved. Regarding Pgm, some individuals present one band whereas others have two. In this particular case it is possible to establish a correspondence between one band and one allele, the homozygotes have one band and the heterozygotes two.

In the course of the experiments four different genotypes were found. They correspond either to one-band zymograms or to two-band zymograms. The different migration levels correspond to three alleles Pgm1, Pgm2, and Pgm3 according to their increasing mobility. The observed genotypic frequencies in a sample of 131 individuals (Table 1) are:

Pgm1//Pgm2 = 4/131 = 0.0305
Pgm2//Pgm2 = 78/131 = 0.5954
Pgm2//Pgm3 = 40/131 = 0.3053
Pgm3//Pgm3 = 9/131 = 0.0687

Two genotypes (Pgm1//Pgm1 and Pgm1//Pgm3) have not been observed in our laboratory population.

The allele frequencies of the sample studied, calculated from the genotype frequencies, are:

[4] Program STAT-ITCF., SESI, Boigneville, F-91720 Maisse, France.

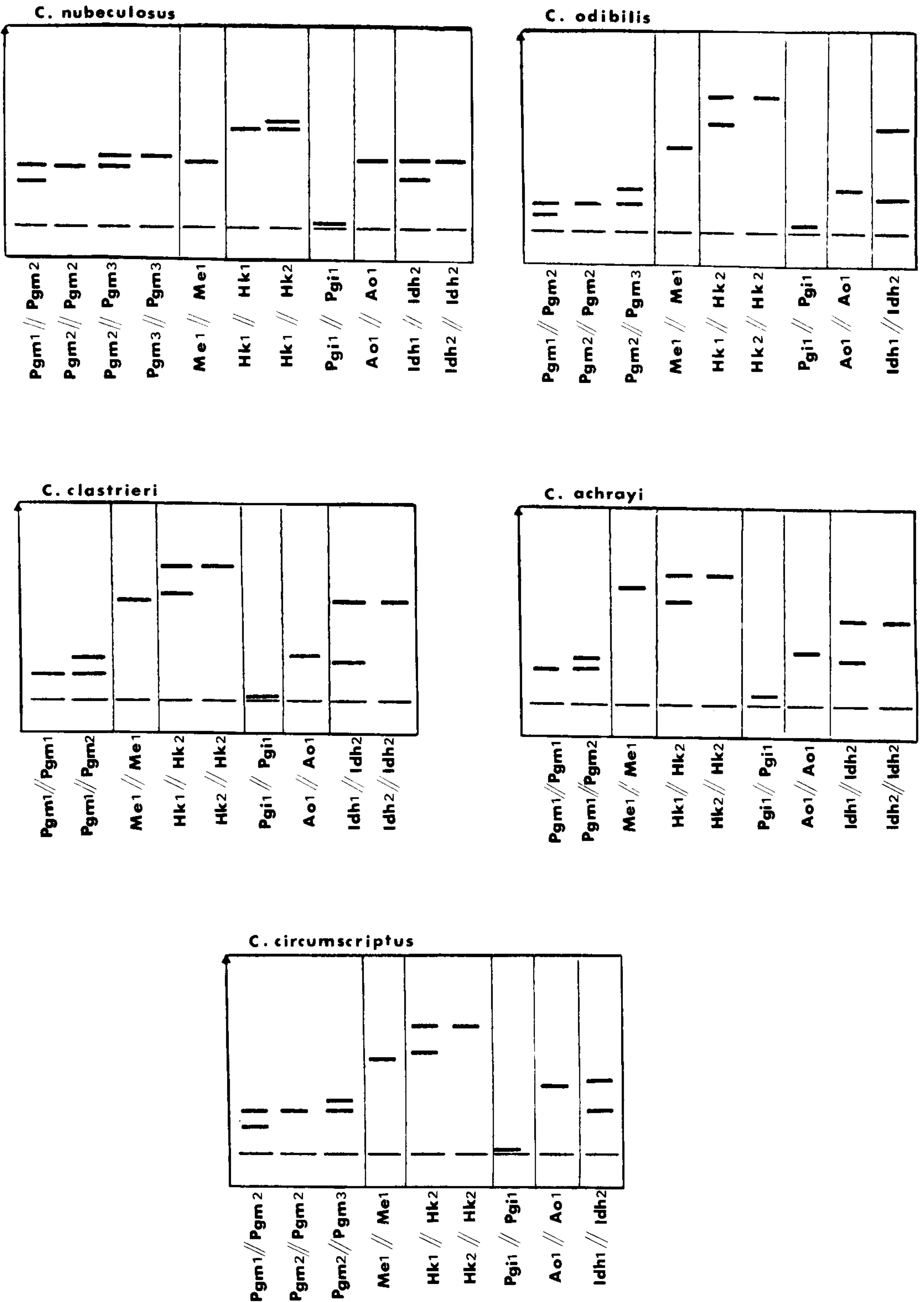

Fig. 1. Juxtaposed zymograms for *Culicoides nuberculosus, C. odibilis, C. clastrieri, C. achrayi* and *C. circumscriptus*.

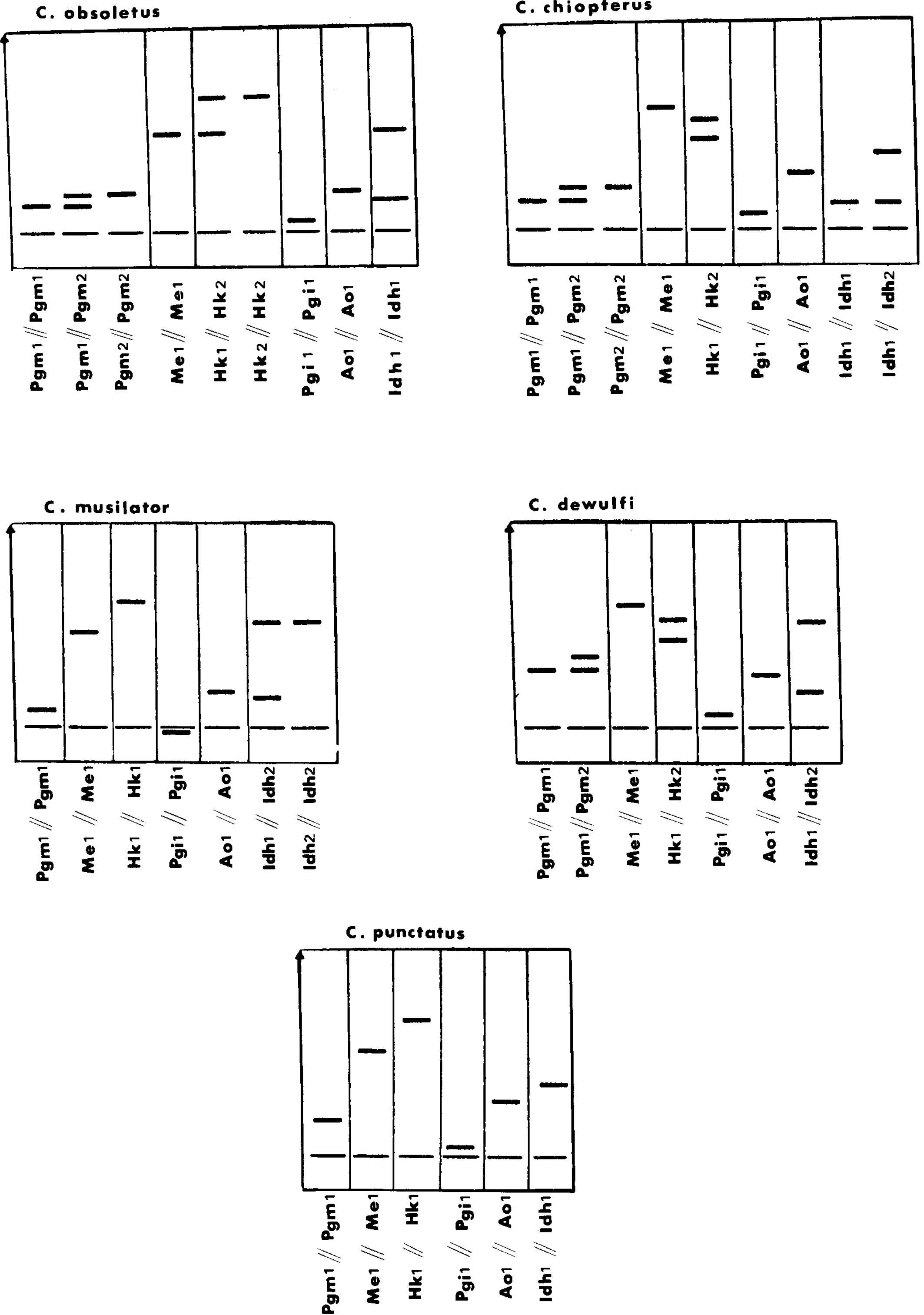

Fig. 2. Juxtaposed zymograms for *Culicoides obsoletus, C. chiopterus, C. musilator, C. dewulfi* and *C. punctatus.*

Table 1. Comparison of the theoretical and observed numbers of the different genotypes to the Pgm locus [after Waller et al. (1986)].

	Genotypes						Total
	Pgm1//Pgm1	Pgm2//Pgm2	Pgm3//Pgm3	Pgm1//Pgm2	Pgm1//Pgm3	Pgm2//Pgm3	
Observed number of different genotypes	0	78	9	4	0	40	131
Observed genotypical frequencies	0.0000	0.5954	0.0687	0.0305	0.0000	0.3053	1
Theoretical genotypical frequencies	$p^2 = 0.0002$	$q^2 = 0.5827$	$r^2 = 0.0490$	$2\,pq = 0.0233$	$2\,pr = 0.0068$	$2\,qr = 0.3380$	1
Theoretical number of different genotypes	0.0305	76.3	6.4	3.1	0.9	44.3	131

$p = 0.0153$ for PGM1
$q = 0.7633$ for PGM2
$r = 0.2214$ for PGM3

Theoretically, the six possible genotypes are:

Pgm1//Pgm1 (not found)	Pgm1//Pgm2
	Pgm1//Pgm3 (not found)
Pgm2//Pgm2	
Pgm3//Pgm3	Pgm2//Pgm3

The data (Table 1, line 1) observed do not differ significantly from the theoretical data (Table 1, line 4) of Hardy-Weinberg (χ^2, $P = 5\%$, NS). This allows us to think that in the insectary, reproduction is panmictic with reference to the character under study.

The random sampling (of n = 131 individuals) did not lead to the Pgm1//Pgm3 genotype. On the opposite, the absence of the Pgm1//Pgm1 genotype can be explained by the fact that theoretically, for this genotype to be revealed the required minimum sample should be approximately 4,300 individuals. This type of research has not yet been carried out for the other enzymes (Hk and Idh).

Correspondence Analysis. Correspondence analysis is defined by de Lagarde (1983) as: "a graphical representation of the data tables. Its purpose is to put together in one or a small number of graphs, as much information as possible from one table, by referring, not to the absolute values but rather to the correspondence between characters, i.e., to the relative values".

We performed correspondence analysis on the previously mentioned electrophoresis results. In order to collect the data, the zymograms were subdivided into levels parallel with the perforations of the gel in such a way that the two bands of a heterozygote be situated in two different sections (Fig. 3).

Five levels were thus obtained for Hk, six for Pgm, four for Me, five for Idh, four for Ao and three for Pgi; thus a total of 27 potential levels. These migration levels are used as a set of features for each species of *Culicoides*. In this way the features serve to elaborate the data matrix. In this binary matrix any present band is coded as 1, any absent band as 0. The 10 species of *Culicoides* are described in the 10 lines of the matrix. The six enzymatic systems studied, subdivided into 27 potential levels, form the 27 columns of the matrix (Table 2).

Correspondence analysis will give us the graphic representations on which the different species of *Culicoides*, as well as the featured electrophoretic markers which differentiated them, are projected. This plane of representation is defined by pairs of axes, each of them being an axis of symmetry of the ellipsoid made

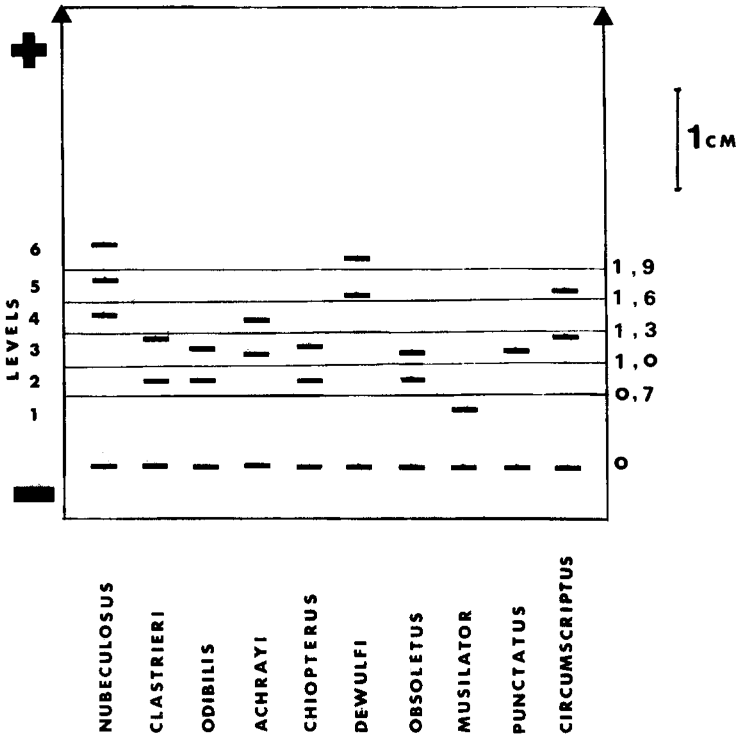

Fig. 3. Phosphoglucomutase (Pgm) enzymatic levels (= characters) for 10 *Culicoides* species. Level 1 lower than 6 mm, level 2 between 7 and 10 mm, level 3 between 11 and 13 mm, level 4 between 14 and 16 mm, level 5 between 17 and 19 mm and level 6 higher than 20 mm.

Table 2. Data matrix obtained from the codage of the observed bands on the different zymograms, for 10 different species of *Culicoides*.

Species	Enzymes	Hk	Pgm	Me	Idh	Ao	Pgi
	Levels	1 2 3 4 5	1 2 3 4 5 6	1 2 3 4	1 2 3 4 5	1 2 3 4	1 2 3
C. nubeculosus		0 1 1 0 0	0 0 0 1 1 1	1 0 0 0	0 1 1 0 0	0 0 0 1	0 1 0
C. clastrieri		0 0 1 0 1	0 1 1 0 0 0	0 0 1 0	0 1 0 0 1	0 1 0 0	0 1 0
C. odibilis		0 0 1 0 1	0 1 1 0 0 0	0 1 0 0	0 1 0 0 1	0 1 0 0	0 1 0
C. achrayi		0 0 1 0 1	0 0 1 1 0 0	0 0 0 1	0 1 0 1 0	0 0 1 0	0 0 1
C. chiopterus		1 0 1 0 0	0 1 1 0 0 0	0 0 0 1	1 0 1 0 0	0 0 1 0	0 0 1
C. dewulfi		1 0 1 0 0	0 0 0 0 1 1	0 0 0 1	1 0 0 0 1	0 0 1 0	0 0 1
C. obsoletus		0 1 0 0 1	0 1 1 0 0 0	0 1 0 0	1 0 0 0 1	1 0 0 0	0 0 1
C. musilator		0 1 0 1 0	1 0 0 0 0 0	0 1 0 0	1 0 0 0 1	1 0 0 0	1 0 0
C. punctatus		0 0 0 0 1	0 0 1 0 0 0	0 0 1 0	0 0 1 0 0	0 0 1 0	0 0 1
C. circumscriptus		0 1 0 1 0	0 0 1 0 1 0	0 1 0 0	0 1 1 0 0	0 0 0 1	0 1 0

0 = no band; 1 = 1 band.

up of the whole *Culicoides* features and individuals. The equation of these axes is obtained by diagonalizing the data matrix.

Given the aforementioned correspondence between electrophoretic bands and alleles, the type of coding used and the resulting graphic representations enabled us to calculate the genetic distances (χ^2 distances) between the different species observed.

On the graph, each *Culicoides* species is symbolized by the first three letters of its name (Figs. 4 and 5), and its enzymatic features are connected to it.

Certain species which are similar in their morphology were compared in this study. This concerns *C. odibilis* and *C. clastrieri* gathered in the *Odibilis* group (species of average size) with few distinguishing features (Callot et al. 1962): coloration of the thorax, slight variations of the pharyngeal ornamentation in the female, a spot

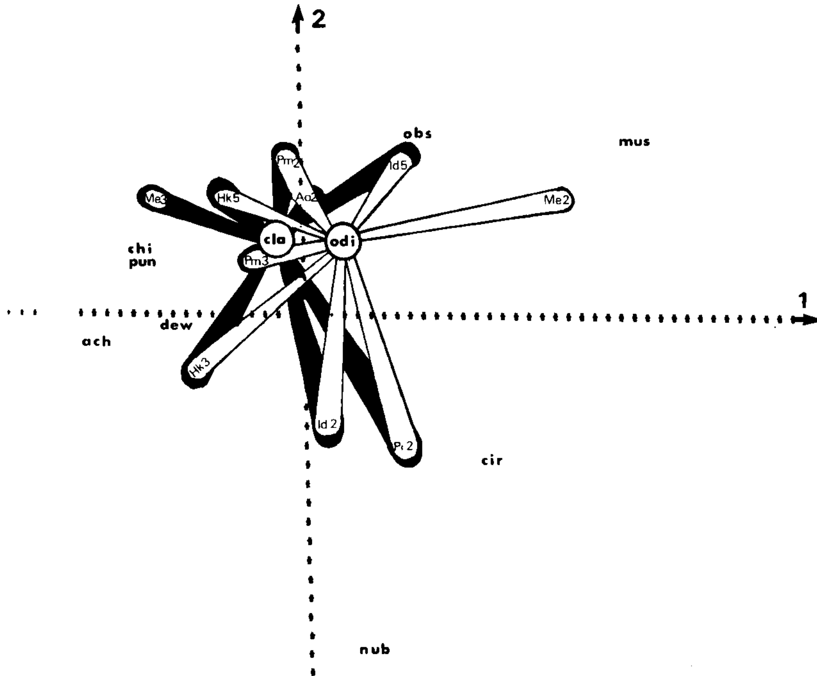

Fig. 4. Plane representation obtained after CFA enzymatic data. Comparison between *Culicoides clastrieri* and *C. odibilis*. *nub* = *C. nubeculosus*, *cir* = *C. circumscriptus*, *mus* = *C. musilator*, *obs* = *C. obsoletus*, *chi* = *C. chiopterus*, *pun* = *C. punctatus*, *ach* = *C. achrayi*, *dew* = *C. dewulfi*, *cla* = *C. clastrieri*, *odi* = *C. odibilis*. Pm = Pgm, Pi = Pgi, Id = Idh.

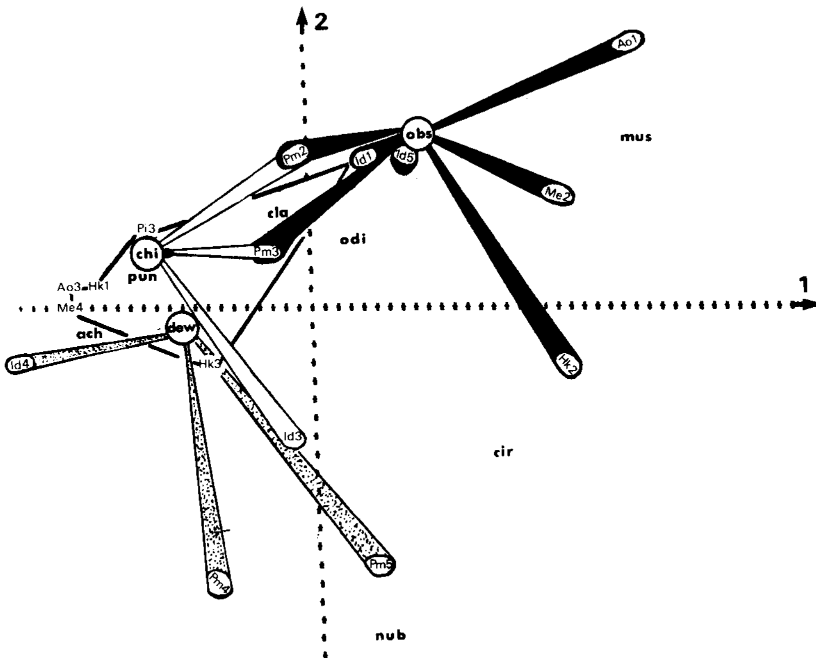

Fig. 5. Plane representation obtained after CFA enzymatic data. Comparison among *Culicoides obsoletus*, *C. chiopterus* and *C. dewulfi*. Abbreviations as in Fig. 4.

on the wings located between the m1 and m2 cells, as well as the distribution of the coeloconic sensillae, particularly in females. Similarly, for three other species, *C. chiopterus, C. dewulfi* and *C. obsoletus* of the subgenus *Avaritia* (species of small and medium size), the basic distinguishing features are the size of the spermathecae, the eyes (which are pubescent in *C. chiopterus*), the shape of the third segment of the palp and the insertion of the subocular hairs in the female (Kremer and Rebholtz 1977). Electrophoretic differences were most often found between the various species analyzed: for example, *C. musilator* is well characterized through Pgm level 1 and Pgi level 1, which are not found in any other single species. Certain species appear to be close in view of our electrophoretic criteria. This is true, particularly for *C. odibilis* and *C. clastrieri*. In the same way, *C. chiopterus* and *C. dewulfi* are similar, but we can observe that *C. obsoletus* acquires its own identity. The results produced by the other plane representations, not shown here, are in agreement with that of axes 1–2. These graphic similarities convey a genetic proximity which we will attempt to refine in the very near future.

DISCUSSION

Biochemistry of Culicoides. We have demonstrated six discriminant enzymatic systems for 10 species from the western zone of the Palearctic region. The results are not exhaustive and the improvement of our techniques should enable us to increase the number of discriminant systems. Our first results outline what the biochemical taxonomy of the *Culicoides* genus will be. To achieve this, it will be necessary to analyze complementary samples to obtain a clear view of the polymorphism of the populations studied. In any case our results will be complemented by the research of all possible genotypes for each species. Mendelian analyses on each enzymatic system must be carried out to confirm their genetic determinism.

Genetic Study of C. nubeculosus. Through the *C. nubeculosus* samples studied by means of Pgm, it appears that the population in our insectary corresponds to a Hardy-Weinberg equilibrium relative to the Pgm locus, there is no preferential pairing and coupling is at random. This enzyme is monomeric in the population of *C. nubeculosus* observed. Three different alleles were identified. Certain genotypes can often be found in the breeding group, whereas other types are rare due to the discrepancy in allelic frequencies. Pgm2 appears often, whereas Pgm1 appears rarely. Phosphoglucomutase is a reliable marker of simple determinism in *Culicoides*.

Processing Data on Zymograms. The processing of the results by means of CFA produces graphic representations of the genetic variability analyzed. Only one graph (axes 1–2, Fig. 4 and 5) is shown here, which represents approximately 50% of the total electrophoretic variability.

This representation enables us to visualize the proximity of the various species. The closer they come together on the graph, the closer they are genetically speaking. It also shows which enzymatic system is responsible for this proximity.

The first results will be supplemented by observation of a maximum number of other species in order to obtain a comparative enzymatic knowledge of each one. A limiting factor of this research is the requirement of 10 individuals per species. As their protein constitution has to remain intact, species coming from other regions must either be transported in liquid nitrogen or obtained at a larval stage, and we have to await emergence in the insectary.

CONCLUSION

The biochemical approach to the genus *Culicoides* enabled us to anticipate a new taxonomical classification and to support our results with a genetic study for at least one species. We can see that a convergence exists between the morphological classification and a classification based on the study of enzymatic polymorphism by electrophoresis. The future prospects of this methodology are promising with regard to the genetic study of populations, their classification, and later their phylogeny when enough species will have been analyzed.

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