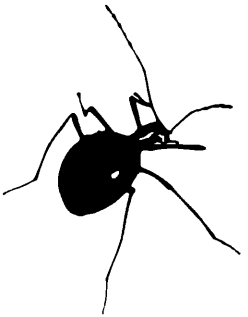

LJUBLJANA, JUNE 2004 Vol. 12, No. 1: 89–98

XVII. SIEEC, Radenci, 2001

# ELECTROPHORETIC PATTERNS OF BODY EXTRACT PROTEINS IN SOME DIPLOPODS (DIPLOPODA, MYRIAPODA)

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**Abstract** - The technique of the universal film agarose electrophoresis has been used to detect biochemical characteristics of the body extract proteins in 7 different species and 7 genera of the diplopod families Julidae, Craspedosomatidae, and Polydesmidae. The migration rate of these proteins as well as their relative abundance for *Julus terrestris* Linnaeus (Julidae), *Melogona broelemanni* Verhoeff (Craspedosomatidae), *Pachyiulus hungaricus* Karsch, *Unciger foetidus* (C. L. Koch), *Leptoiulus trilineatus* (C. L. Koch), *Megaphyllum unilineatum* (C. L. Koch) (Julidae), and *Brachydesmus dadayii* Verhoeff (Polydesmidae) were compared. Electrophoretic identifications of these species showed both species-specific and (probably) intergeneric differences. It is assumed that body protein electrophoresis can be also used: (a) to differentiate species in any stage of their life cycle, (b) to reveal the presence of sibling species, and to differentiate the taxonomic and evolutionary interrelations both in congeneric as well as in other, more distant taxa.

KEY WORDS: protein electrophoresis, species identification, speciation, higher taxa, diplopods

**Izvleček** – ELEKTROFOREZNI VZORCI PROTEINOV TELESNIH IZVLEČKOV NEKATERIH DVOJNONOG (DIPLOPODA, MYRIAPODA)

Za ugotavljanje biokemičnih značilnosti proteinov telesnih izvlečkov 7 različnih vrst in 7 rodov dvojnonog družin Julidae, Craspedosomatidae in Polydesmidae smo

uporabili tehniko splošne tankoplastne gelske elektroforeze. Primerjali smo stopnje selitev proteinov in njihovo relativno pogostnost pri vrstah *Julus terrestris* Linnaeus (Julidae), *Melogona broelemanni* Verhoeff (Craspedosomatidae), *Pachyiulus hungaricus* Karsch, *Unciger foetidus* (C. L. Koch), *Leptoiulus trilineatus* (C. L. Koch), *Megaphyllum unilineatum* (C. L. Koch) (Julidae) in *Brachydesmus dadayii* Verhoeff (Polydesmidae). Elektroforezne določitve teh vrst so pokazale vrstno specifične in (verjetno) medrodovne razlike. Predvidevamo, da je elektroforeza telesnih proteinov uporabna za (a) razločevanje vrst v katerem koli razvojnem stadiju, (b) razkrivanju prisotnosti sestrskih vrst in ugotavljanju taksonomskih in evolucijskih odnosov v okviru rodov, kot tudi med bolj oddaljenimi taksoni.

KLJUČNE BESEDE: elektroforeza proteinov, vrstna določitev, razvoj vrst, višji taksoni, dvojnonoge

## Introduction

Electrophoretic methods have clarified taxonomic relationships in several arachnid and insect groups (e.g., Ayala and Powell, 1972; May *et al.* 1977; Vachon and Goyffon, 1978). These methods have an advantage over morphological methods in that they can be applied to any arachnid or insect stage without regard to morphological condition, so long as the soluble proteins do not become denaturated. Recently, Berlocher (1984) reviewed the literature in biochemical taxonomy in insects and enumerated alternate analytical approaches. While these methods have not been yet applied to diplopods, biochemical profiles have been provided for numerous species complexes and a number of higher taxa of insects (Berlocher, 1984). The above studies clearly demonstrated the value of biochemical characters as tools for species identification.

The use of biochemical characters in evolutionary zoology has a long history, and there are a number of excellent reviews of the field (Hawkes, 1968; Wright, 1974; Shishiniova, 1976; Shishiniova and Vassilev, 1985; Saul *et al.* 1977; Berlocher, 1984; Ćurčić *et al.* 1994). If we accept that the most useful taxonomic characters should be those that are relatively free of environmental and other nongenetic influences, than it follows that among biochemical compounds, those that are most closely linked to the genome (such as specific proteins), should show the greatest value. This implies a biochemical hierarchy with a close correspondence with systematic usefulness. In practice, however, knowledge of this hierarchy has had little impact on the way in which biochemical characters are chosen and used in taxonomy, the decisions being made on empirical ground.

There are two potential problems that arise in species, generic, and even familial identification of diplopods. The first involves the ever-present problem of cryptic species complexes, *i.e.* biologically distinct species that are nevertheless so similar morphologically that their distinctiveness is not realized. The second occurs where closely related taxa are morphologically distinguishable at some instars and

not at others, yet all stages need to be identified in the field situation. Both of these problems are amenable to analysis by the protein electrophoresis of body extracts, but as yet have received no attention from myriapodologists.

Evolutionary zoologists have applied biochemical techniques not only to the differentiation of taxa but also to produce far-reaching conclusions concerning evolutionary processes, biological clocks, and phylogenetic trees (Cain, 1983). In this study, however, we focus on a much narrower taxonomic problem. In view of the special problem of the taxonomy of diplopods, and their relevance to control (Ćurčič and Makarov, 1995), it is perhaps surprising that practically no attention has been devoted to potential biochemical characters. In this paper we shall briefly discuss the particular problems and importance of generic identification in seven sympatric representatives of diplopods: *Julus terrestris*, *Melogona broelemanni*, *Pachyiulus hungaricus*, *Unciger foetidus*, *Leptoiulus trilineatus*, *Megaphyllum unilineatum,* and *Brachydesmus dadayii,* and then examine a biochemical approach that appears to hold particular promise in providing taxonomic character.

## Material and methods

Live adult specimens of the seven species and seven diplopod genera were collected during September 2000 by sieving humus and leaf-litter in an oak and beech forest, from Mt. Avala, near Belgrade, Yugoslavia. The species identification was verified at the adult stage.

The live specimens of each species and genus were kept in separate vials, at + 8°C for three days for acclimatization in the laboratory (Ćurčić, 1994); these were isolated from each other in order to avoid damage and/or cannibalism. The diplopods were also exposed to the starvation diet for three days in order to avoid the interference of food ingestion with electrophoretic analysis of body extracts.

The electrophoresis was performed on the Universal Electrophoresis Film Agarose (*Corning Diagnostics Corp.*, Palo Alto, CA, USA), otherwise used for analysis of body proteins. The film components were as follows: 1% (w/v) agarose, 5% (w/v) sucrose, 0.0035% (w/v) EDTA disodium, and 0.065 M barbital buffer.

The samples of *Julus terrestris*, *Melogona broelemanni*, *Pachyiulus hungaricus*, *Unciger foetidus*, *Leptoiulus trilineatus*, *Megaphyllum unilineatum,* and *Brachydesmus dadayii* were prepared for electrophoresis by grinding each sample (100 mg each) into 100 µl ice cold 0.9% sodium chloride. After 20 min of centrifugation at 13,800 rpm (*Beckman* centrifuge), 0.8 µl of supernatant of each species and generic sample was transferred by capillary action to the marked origin (or a separate horizontal slit) of the film. Four applications per sample were made to the agarose film using p200 *Pipetman* disposable tip. The protein electrophoresis was conducted at a current of 90 V with a running time of 35 min in the electrophoretic vessel (containing the barbital buffer, titrated to pH 8.6), until the "front" had migrated cca 40 mm. After protein fractionating in the electric field, each film was stained with Amido Black 10B as the tracking dye (*Pfizer Diagnostic Division*, New York,

N. Y., USA) for general proteins. The destaining was performed for 15 min in 5% acetic acid. The agarose films were then dried for 20 min at 50°C; subsequently, an additional destaining was performed by rinsing each film for 5 min in 5% acetic acid and eventually the films were dried for 20 min at 50°C. Simultaneusly, the analysis of normal human serum (control normal serum, *Corning Diagnostics Corp.*, Palo Alto, CA, USA) was used as a control, since no adequate electrophoretic patterns have been known for diplopods.

Under such conditions, soluble proteins in the sample move at a rate and direction according to their molecular weight and electric charge, which in turn are determined primarily by their amino acid composition. Therefore, the protein fractions were identified electrophoretically; and the relative abundance was determined by means of a densitometer (*Sebia*, Issy, France).

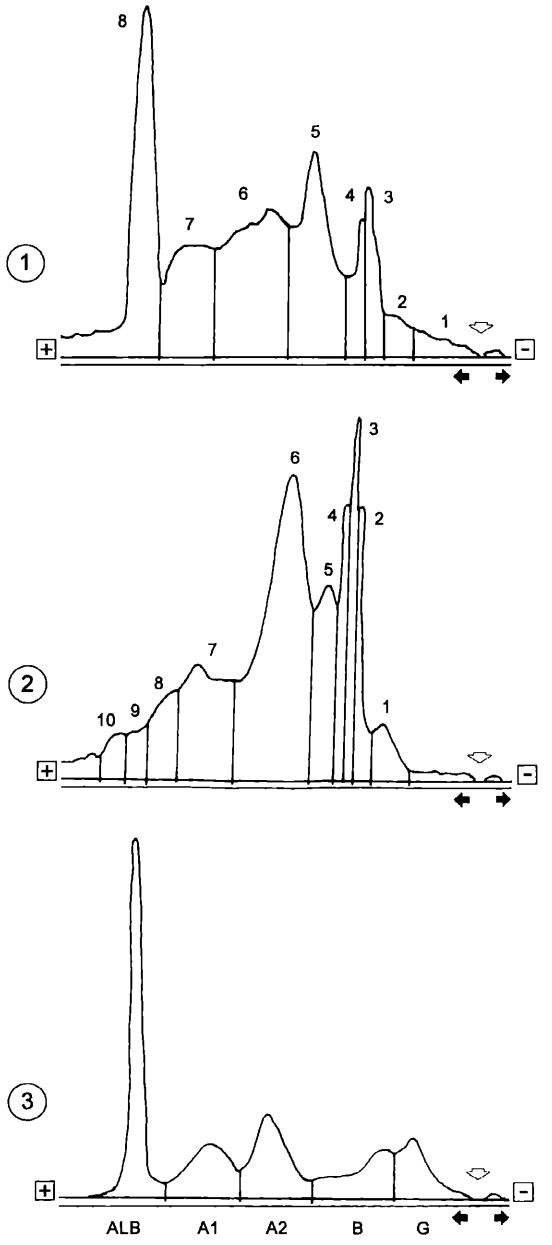
## Results and discussion

By the methods mentioned above, the body extract in the diplopods studied was found to contain different protein components denoted as fractions 1-12 (Figs. 1, 2, and 4-8). The qualitative and quantitative distinctions in the body extract proteins were revealed in all analyzed diplopod species (Figs. 1, 2, and 4-8).

The densitogram of *Julus terrestris* (Fig. 1) is characterized by eight protein fractions (1-8) which exhibit considerable differences in their relative abundance; fractions 1-4 and 6-7 are more conspicuous, while fractions 5 and 8 are more prominent. The two fractions (1, 2) correspond to the "level" of the human G-globulins; however, fractions 3 and 4 correspond to the "migration" of human B-globulins, fraction 5 to the level of human A2- and B-globulins, fractions 6 to the level of human A1- and A2-human globulins, fractions 7 to the migration of human A1-globulins, and fraction 8 to the level of human albumins (Figs. 1 and 3).

The sample of the body extract proteins in *Pachyiulus hungaricus* (Fig. 2), clearly showed that the most distant from the start is fraction 10, which is more mobile that human albumins. However, the fraction 6 has the highest protein content; it migrates to the level of human A2-globulins. Fractions 9 and 10 are predominantly at the level of human albumins, fractions 7 and 8 mostly at the level of A1-albumins, while fractions 2-5 are at the level of human B-globulins; finally, fraction 1 is found on the border between human B- and G-globulins (Figs. 2 and 4).

Figure 4 represents the electrophoretic pattern of the body extract proteins in *Unciger foetidus*. This densitogram is characterized by 4 protein fractions (1-4) which exhibit considerable differences in their relative abundance; fractions 1 and 2 are most outstanding (highest), while fractions 3 and 4 are less prominent (Fig. 4). The fraction 1 corresponds to the level of human B-globulins, and partially to the migration of human A2-globulins; the following fraction (2) corresponds to the level of human A1- and A2-globulins; fraction 3 migrates similarly to human albumins and A1-globulins, while fraction 4 is found distally to the level of human albumins. The most intensive precipitation in this species and genus correspond to the migration of human A1-, A2- and B-globulins (Figs. 3 and 4)




**Figs. 1-3:** Protein electrophoresis pattern of body extracts in *Julus terrestris* Linnaeus (Fig. 1), *Pachyiulus hungaricus* Karsch (Fig. 2), and of human normal serum (Fig. 3). Open arrows represent film slits and solid arrows indicate the direction of migration of protein fractions.

The study of the body extract proteins in *Leptoiulus trilineatus* (Fig. 5) revealed some qualitative distinctions in comparison to electrophoretic patterns of both *U. foetidus* and *M. unilineatum* (Figs. 4 and 6). In *L. trilineatus*, the "travel" of the majority of protein fractions is shorter than that of human albumins (Fig. 3). Here, the fastest migrating proteins consist of two distinct fractions (2 and 3), which correspond to the human A1- and A2-globulins, respectively. Fraction 1 corresponds to the level of human B-globulins, while fraction 4 is at the level of human albumins (Fig. 3). Therefore, it is evident that this electrophoretic pattern is clearly distinct from that in *U. foetidus* (Fig. 4).

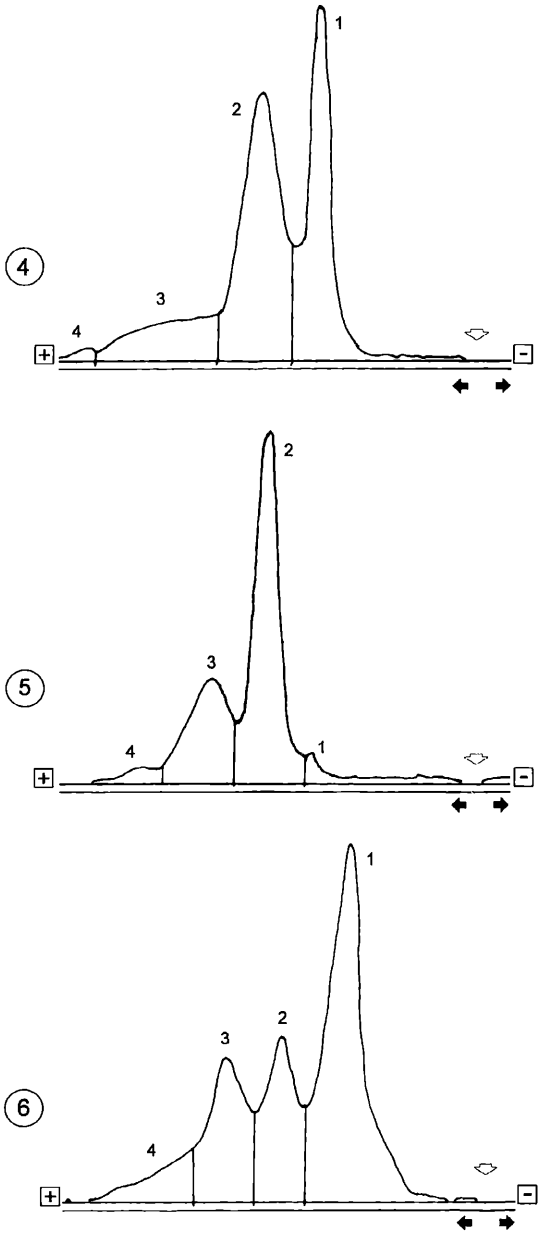
The study of the body extract proteins in *M. unilineatum* (Fig. 6), clearly showed that the most distant from the start is fraction 4, which is as mobile as human albumins (and A1-globulins; Figs. 3 and 6). In addition, the fraction 1 has the highest protein content; this fraction migrates up to the level corresponding to human B-globulins (and, partially, to that of G-globulins). However, fractions 2 and 4 correspond to the migration of human A2-globulins and both A1- and A2-globulins, respectively (Figs. 3 and 6). Evidently, the electophoretic protein pattern of *M. unilineatum* is clearly distinctive in relation to those of either *U. foetidus* or *L. trilineatus*.

The study of the body extract proteins in *Melogona broelemanni* (Fig. 7) revealed some qualitative distinctions in comparison to electrophoretic pattern of the preceding taxon. In *M. broelemanni*, the "travel" of the majority of protein fractions is shorter than that of human proteins (Figs. 7 and 9). Here the fastest migration proteins consist of fractions 4 and 5, which correspond to human A2- and A1-globulins, respectively. At the level of human B-globulins there are fractions 2 and 3 while a small fraction 1 is related to the "migration" of human G-globulins (Figs. 7 and 9).

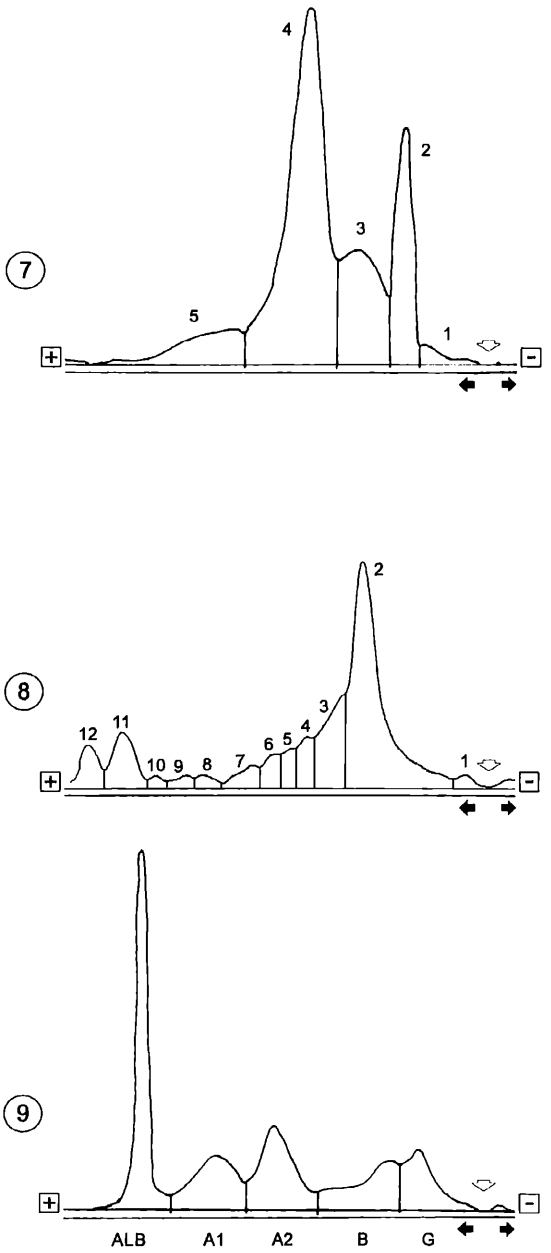
Figure 6 represents the electrophoretic pattern of the body extract proteins in *Brachydesmus dadayii*. This densitogram is characterized by 12 protein fractions (1-12) which exibit clear differences in their relative abundance; fractions 11 and 12 migrated distally to the level of human albumins; fractions 8 and 9 correspond to the level of A1-globulins, 4-6 to the niveau of human A2-globulins, while fractions 2 and 3 correspond to the niveau of human B- and G-globulins (Figs. 8 and 9), where the most intensive precipitation of this species and genus is found.

The outstanding distinctions in densitograms of the seven analyzed diplopod species and genera support the view that the confamilial genera of diplopods are much more similar than the higher taxa pertaining to different families (Chordeumatidae; Boškova *et al.* 2000). However, one of the main goals for the future research is to find out whether the supposed interspecific, intergeneric and interfamilial distinctions are universally present in other both lower and higher taxa of diplopods.

It is evident that the electrophoretic patterns of body extract proteins in the seven diplopod species and genera are clearly distinctive (Figs. 1, 2 and 4-8); additionally it can be also seen that the representatives of the family Julidae are electrophoretically much more similar than is each of these compared to the members of either Chordeumatidae or Brachydesmidae.




**Figs. 4-6:** Protein electrophoresis pattern of body extracts in *Unciger foetidus* (C. L. Koch) (Fig. 4), *Leptoiulus trilineatus* (C. L. Koch) (Fig. 5), and *Megaphyllum unilineatum* (C. L. Koch) (Fig. 6). Open arrows represent film slits and solid arrows indicate the direction of migration of protein fractions.




**Figs. 7-9:** Protein electrophoresis pattern of body extracts in *Melogona broelemanni* Verhoeff (Fig. 7), *Brachydesmus dadayii* Vehoeff (Fig. 8), and of human normal serum (Fig. 9). Open arrows represent film slits and solid arrows indicate the direction of migration of protein fractions.

Diplopod populations that are not reproductively isolated will tend to remain biochemically (and genetically) uniform; indeed theoretical studies suggest that even very low rates of gene flow between populations will maintain this genetic inertia (Kimura and Weiss, 1974). However, once the populations are reproductively isolated, the opportunity arises to escape this genetic inertia and the populations are able to diverge.

In this study, film agarose electrophoresis clearly separated the sympatric species which are, by all means, reproductively isolated. Biochemical (and genetic) divergence in isolation will result from two processes - change in allele frequencies of loci already polymorphic in the original population, and the accumulation by mutation of different alleles in the different populations. The result is that with progressive time in isolation, the populations will become fixed for different alleles at more and more loci (White, 1978).

A problem frequently encountered in diplopod biology is that species morphologically differentiable at one stage of the life cycle (usually the adult) are nevertheless morphologically indistinguishable at other stages of the life cycle. However, protein electrophoresis can he used to distinguish such forms since the main kinds of proteins stained for in a typical electrophoretic analysis are usually constant between different stages of the life cycle (Marshall and Detsch, 1950). This is not suprizing since most such proteins are involved in intermediary metabolism such as glycolysis. Therefore loci that show fixed differences between species at one stage of life cycle will show the same fixed differences at other stages. These loci can therefore be used in the same manner as conventional diagnostic characters, but with the additional advantage that they are applicable to all life stages of the species and that they have a simple known genetic basis.

Sibling species that fail to interbreed in nature (when given opportunity to do so) are good biological species, but are morphologically so similar that they are not (easily) recognized as distinct species. It might be thought that sibling species are not common. However, even among vertebrates, sibling species are being discovered at an alarming rate. One could reasonably anticipate that the incidence of sibling species among arthropods (including diplopods) would be much higher. Failure to recognize such "cryptic" species complexes will invalidate any work that is done on the assumption that only one species is involved, e.g. ecology, physiology, behaviour etc. (Ayala et al., 1975). It is therefore critical that such situations be clarified, and again protein electrophoresis is the tool of choice.

Protein electropboresis has enormous potential for the taxonomic identification of diplopod species, both where certain life stages are difficult to distinguish and where cryptic species are involved. In addition, the biochemical approach in taxonony seems of greatest value not only where speciation has occurred in the absence of much morphological variation, but also in higher taxa (above the species level).

Electrophoretic identification of the studied species of diplopods was useful in providing a check on morphological identifications. Should electrophoretic techniques become more commonly used, they will be powerful tools during outbreaks of diplopod- or arachnid-borne diseases, providing better estimates of vector populations with-

out requiring drastic modification of collection methods. Moreover, electrophoretic identifications may prove useful in meaning the impact of pesticide applications on the size of later adult populations of a particular diplopod species.

Finally, electrophoretic separation of morphologically simillar adults of the analyzed species is reliable and accurate if details of the buffer and pH are carefully controlled. Undeniably, this approach is more expensive and time-consuming in terms of work-hours per diplopod sample (or specimen) identified. Specialized equipment must be purchased or constructed, and time training in the techniques is required. Even so, if information on the presence or density of a medically important species cannot be obtained using conventional methods, the electrophoretic approach may be the method of choice.

## References

**Ayala, F. J., J. R. Powell.** 1972. Allozymes as diagnostic characters of sibling species of *Drosophila. Proc. Natl. Acad. Sci.*, **69**, 1094-1096.

**Ayala, F. J., M. L. Tracey, D. Hedgecock, R. C. Richmond.** 1975. Genetic differentiation during the speciation process in *Drosophila. Evolution*, **28**, 576-592.

**Berlocher, S. H.** 1984. Insect mollecular systematics. *Ann. Rev. Entom.*, **29**, 403-433.

**Cain, A. J.** 1983. Concluding remarks. Pp. 391-397 in G. S. Oxford and D. Rollinson, Eds., Protein polymorfism: adaptive and taxonomic significance. Academic Press, New York and London.

**Hawkes, J. G. (Ed.).** 1968. Chemotaxonomy and serotaxonomy. Academic Press, New York and London.

**Kimura, M., G. H. Weiss.** 1974. The stepping stone model of population structure and the decrease of genetic correlation with distance. *Genetics*, **49**, 561-576.

**Marshall, M. E., H. F. Detsch.** 1950. Protein changes in fluids of the developing chicken embryo. *J. Biol. Chem.*, **185**, 155-161.

**May, B., L. S. Bauer, R. L. Vadas, J. Granettt.** 1977. Biochemical genetic variation in the family Simuliidae: electrophoretic identification of the human biter in the isomorphic *Simulium jenningsi* group. *Ann. Entom. Soc. Am.*, **70**, 637-640.

**Poinar, G. O., Jr., B. P. M. Ćurčić.** 1992. Parasitism of pseudoscorpiones (Arachnida) by Mermithidae (Nematoda). *J. Arachnol.*, **20**, 64-66.

**Poinar, G. O., Jr., B. P. M. Ćurčić.** 1994. Nematode (Nematoda) associate and parasites of pseudoscorpions (Arachnida). *J. Inv. Pathol.*, **63**: 95.-96.

**Saul, S. H., P. R. Grinstad, G. B., Jr., Craig.** 1977. Identification of *Culex* species by electrophoresis. *Am. J. Trop. Med. Hyg.*, **26**, 1009-1012.

**Vachon, M., M. Goyffon.** 1978. Chimiotaxonomie: valeur et limites des caractères tirés du protéinogramme de l'hémolymphe en gel de polyacrylamide de differentes espèces de scorpions. *Symp. zool. Soc. Lond.*, **42**, 317-325.

**White, M. J. D.** 1978. Modes of speciation. Freeman, San Francisco.

**Wright, C. A., (Ed.).** 1974. Biochemical and immunological taxonomy of animals. Academic Press, New York and London.