Michael Hubbard

Proc. IVth Intern.Confer.Ephemeroptera
V.Landa et al.(eds.),213-218,ČSAV,1984

APPLICATION OF THE SCANNING ELECTRON MICROSCOPE AND THE ENZYME-GEL-ELECTROPHORESIS TO SOLVE TAXONOMICAL PROBLEMS: THE EUROPEAN SPECIES OF THE GENUS EPEORUS SENSU TSHERNOVA (1981) (EPHEMEROPTERA, HEPTAGENIIDAE)

A. Zurwerra, I. Tomka and G. Lampel

Zoologisches Institut der Universität Freiburg, Entomologische Abteilung, CH-1700 Freiburg, Switzerland

Abstract. Eleven populations from 70 investigated localities of the European Epeorus-taxa were studied by scanning electron microscopy /surface structures/ and by enzyme electrophoresis. The biochemical data correlate well with the morphological characters. The larvae of E. torrentium and E. sylvicola differ in the pilosity of the inner edge of the glossae. Four of the 17 enzymes separated on vertical starch gel were identical in their mobility, the other 13 clearly indicated interspecific differences. Based on Nei's coefficient of genetic identity there are two distinct clusters in the dendrogram: /1/ E. alpicola - E. yougoslavicus /$\bar{I}$ = 0.64/ and /2/ E. torrentium - E. sylvicola /$\bar{I}$ = 0.86/. The applied methods will be used to other taxa of the Heptageniidae.

Biosystematics, methods, genetics similarities

This paper is a preliminary report of the first results of our research work done with the family Heptageniidae. We have chosen the genus Epeorus Eaton 1881, as initial group because of the rather simple external morphology. In addition to the usual morphological methods we have applied the scanning electron microscopy (Tomka and Hasler 1978) and the enzyme electrophoresis (Zurwerra and Tomka 1982) for the first time in ephemeropterology. Our aim was to see whether biochemical and morphological data correlate or not.

Puthz (1978) listed 5 Epeorus-species in the Limnofauna Europaea: E. alpicola Eaton, 1871), E. caucasicus (Tshernova,

*Research supported by the Swiss National Science Foundation Grant No. 3.676 - 0.1980

1938), E. sylvicola (Pictet 1865), E. torrentium Eaton 1881, and E. yougoslavicus (Šámal 1935). Four of them we investigated with our methods. The material was collected by us in Europe at 70 localities, mainly mature larvae. For the biochemical separation it was necessary to have imagines of both sexes. Therefore we transported living specimens in dewar containers or in cooling tanks home and reared them in the laboratory until they reached the stage of the imago. The wings and the final abdominal segments of most female and male imagines were cut. The rest of each imago was stored separately at - 70°C until using it in the enzyme electrophoresis. The cut parts of the imagines and even whole specimens were conserved in 80% alcohol, which were used for taxonomical determination later on. The larval mouth parts, gills, legs and skins were mounted into a polyvinylalcohol-lactophenol-mixture on slides in order to compare and to determine them by using an optical microscope. Wings and other parts of the subimagines and imagines were given the same treatment. In order to take pictures of penes and eggs of the different species we used the scanning electron microscope.

To achieve an image formation with elastically scattered electrons it is necessary to work in high vacuum. Therefore it was essential to eliminate all volatile components of the sample. There are 3 different possibilities to dry a specimen. Air-drying of a water-containing sample always causes damages in the soft tissue of the sample-surface-structures. One has 2 principal ways to eliminate this deformation:

- To keep the sample below its glass transition temperature during the drying process (freeze-drying) or
- to eliminate the surface tension of the sample during the drying process (critical point drying).

The first way is time consuming (low diffusion constant of the water) and may produce cracks if the sample contains material with very different thermo-mechanical properties. Therefore we chose the 2nd way. One may eliminate the surface tension by passing the critical point of the swelling liquid. After having fixed the sample with glutaraldehyde we substituted the water with a range of liquids (alcohol and acetone) ending with liquid carbon dioxide. CO_2 has its critical point at 28° and 80 bar which can easily be realised in a small equipment.

Speciation finds its expression in the genetic material. It is not possible through reliable routine methods to show directly this change in the genetic material. However it is possible to prove 30 - 40% of this genetic alternation (Nei 1971) by the detection of the primary products of the genetic entities (loci). Enzymes are easily identifiable primary products of the gene loci. It must be kept in mind that an enzyme may be produced by more than one gene locus. If this is the case, we say that an enzyme "contains" more than one allele.

The enzymatic investigations on 12% starch gels have been carried out according to the standard methods (Ayala et al. 1972; Scholl et al. 1978). The Ephemeroptera turned out to be

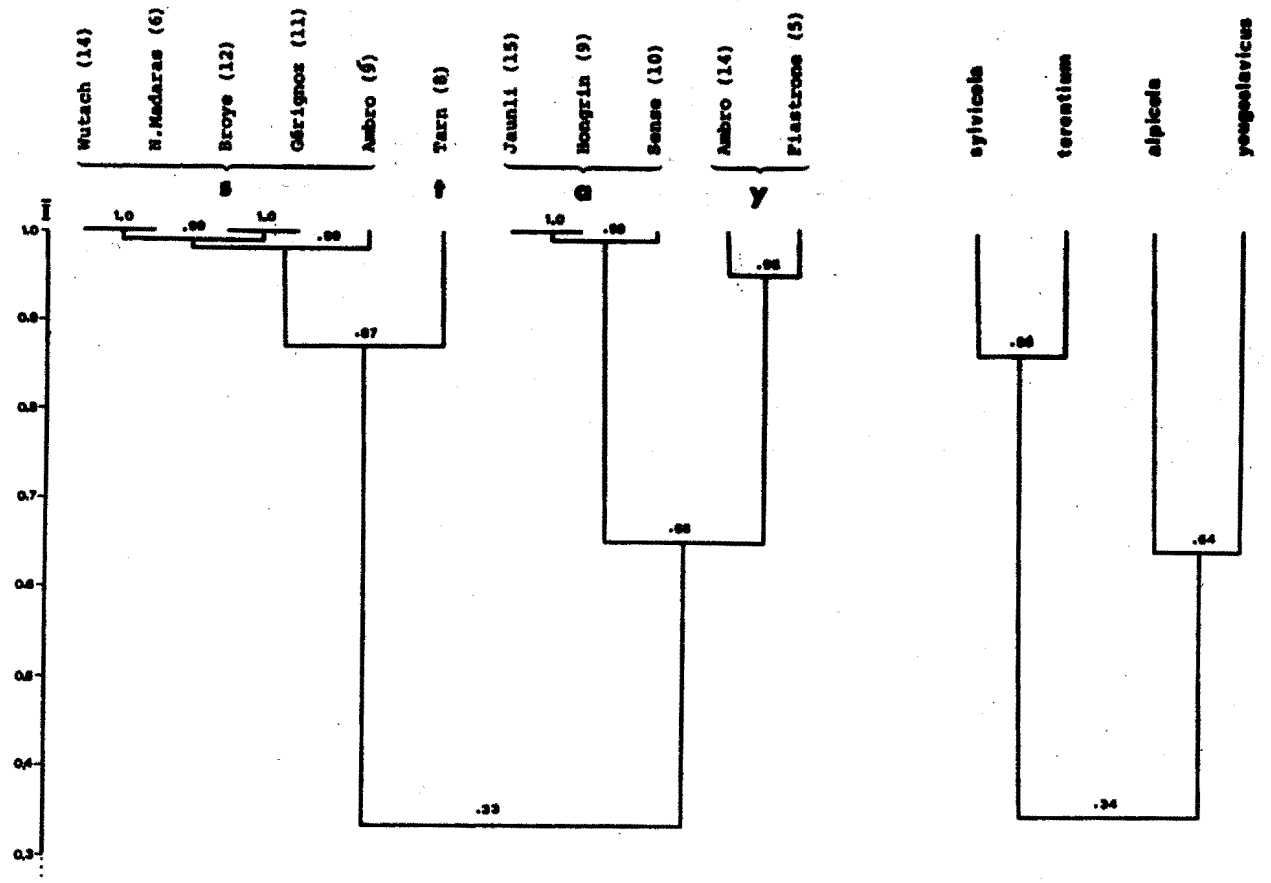

Fig. 1: Dendrogram of the 4 European Epeorus - taxa. / /= Number of biochemically separated animals, I = mean genetic identity /coefficient of the correlation matrix/. The 11 populations originate from Switzerland / Broye, Gérignoz, Hongrin, Jaunli, Sense/, West Germany /Wutach/, France /Tarn/, Italy /Ambro, Fiastrone/ and Rumania /N. Madaras/.

a group which was hard to differentiate by the enzyme electrophoresis. We were able to use only 13 different enzyme-substrate systems for the biochemical separation of the Epeorus-species although we had investigated 42 different enzyme-substrate systems by means of different buffers. With this 13 enzyme-substrate systems we could establish for the 4 Epeorus-taxa 17 different enzyme loci. We investigated the following enzymes: Adenylate kinase (*AK), Arginine kinase (*APK), Aldolase (*ALD), Glutamate-oxaloacetate transaminase (*GOT-1 and *GOT-2), α - Glycerophosphate dehydrogenase (*α-GPDH), Indophenol oxydase (*IPO-1 and *IPO-2), Hexokinase (*HK-1 and *HK-2), Leucine amino peptidase (**LAP), Lactate dehydrogenase (**LDH), Malate dehydrogenase (*MDH-1 and *MDH-2), Mannose phosphate isomerase (**MPI), Phosphoglucomutase (*PGM) and Retinol dehy-

drogenase (**RDH).

The separation of the enzymes was carried out at 12V/cm on vertical starch gels during a period of 5 1/2 - 8 hrs according to the buffers used. We applied the following buffers:

* N-(3-Aminopropyl)-morpholine-citrate (Clayton and Tretiak 1972),
** Tris borate EDTA buffer, pH 9.0 (Ayala et al. 1972).

The 1st buffer was modified and used at 2 different pH values: pH 6.0 (for AK and APK only) and pH 7.0 of the same concentration (Gel: 1mM citric acid, electrode: 20 mM citric acid; buffers are adjusted to correct pH with N-(3-Aminopropyl)-morpholine).

After the electrophoretic run in the refrigerator at 4°C the starch gels were cut horizontally and the resulting halves were stained by means of a generally very selective enzyme-substrate reaction. This reaction - due to its catalytic nature - is associated with an amplification factor of the order 10^4, which is needed if one works with small samples. We separated the proteins of homogenized individuals of imagines of both sexes in order to be able to separate different alleles of the same gene locus. The basis for this separation is the difference in electrical mobility for proteins of a very similar chemical structure. One or two differing amino acids in a sequence of 1000 are sufficient if one selects the proper buffer system.

The distances of the positions of the different alleles from the starting slots were measured and the mobilities relative to a reference allele were determined. We chose at will a population of E. sylvicola as reference. For each enzyme the designation of all electrophoretic variants (electromorphs) was made by comparison with the most frequent variant which defined the relative mobility index 100. The relative mobilities of the 17 different enzymes and their frequencies found in this way are compared in pairs (correlation analysis) within the 4 Epeorus species. The results is the correlation matrix which forms the basis for the construction of the dendrogram (see Fig. 1) according to the unweighted pair-group arithmetic average (UPGMA) clustering method (Ferguson 1980). In the dendrogram genetic similarities (Nei's coefficient of genetic identity (I)) between populations and taxa can be seen easily.

Within the 4 European Epeorus-species we investigated larval (inner edge of glossa, labrum, 1st and 7th gill, number of teeth on the claws) and adult parts (sternal and lateral pattern of the abdominal segments, subgenital plate, pygidium of the female imago, penis of the male imago as well as the egg surface).

Eggs and penes were investigated with the scanning electron microscope. These characters illustrate the close relationship between E. sylvicola and E. torrentium, which are clearly distinct from E. alpicola and E. yougoslavicus. Contrary to Berthélemy and Thomas (1967) we were able to separate also the larvae of E. sylvicola and E. torrentium. The differ-

ence between these 2 species is the pilosity of the inner edge of the larval glossa. There are thin hairs which can be either short or long in E. sylvicola. These hairs cover the inner edge of the glossa up to 2/3. In E. torrentium however, there are long thin hairs and short thick ones which cover about 1/2 of the inner egde of the glossa.

We took at random 11 populations from our 70 localities for the biochemical separation of the 4 European taxa. The homogenate of one individual was sufficient for the enzymatic separation of the 17 loci at one time. About 10 individuals per population and taxon are needed for a biochemical comparison. The mobilities of the proteins determined by this 17 independant enzyme loci were compared within the 4 Epeorus-taxa. Four out of the 17 enzymes electrophoretically separated were identical in their mobility (APK, α-GPDH, MDH-2, MPI). Other monomorph enzymes clearly indicated interspecific differencies in their mobility e.g. AK, ALD, GOT-2, IPO-2, HK-2, and LAP. Polymorph enzymes were GOT-1, IPO-1, HK-1 and PGM. Some electrophoretic variants, however, were rare. Details were published elsewhere. We only show here the dendrogram. The left part of it (Fig. 1) shows the 11 populations. The right part arranges them in their 4 species. Populations of the same species show an $\bar{I}$-value of about 1, because their alleles, with the corresponding frequencies, are more or less equal (see populations 1-5, 7-9, 10-11). $\bar{I}$-values of different species give the percentage of discernible phenotypes.

The morphological and biochemical data show both the grouping of the 4 investigated species of Epeorus: on the one hand E. sylvicola and E. torrentium and on the other E. alpicola and E. yougoslavicus. The $\bar{I}$-values of the genetic identity may be considered as follows:

- Populations which belong to the same species have an $\bar{I}$-value of about 1 according to their definition.
- E. sylvicola and E. torrentium are separated at a level which is normally found with taxa closely related to the same species-group e.g. in the Ecdyonurus helveticus-group (results not published yet).
- E. alpicola and E. yougoslavicus are separated at a level which is common for well defined species.

We applied the genus Epeorus according to Tshernova (1981). Contrary to Braasch (1980) we did not place E. alpicola and E. yougoslavicus into the genus Iron, although the index of the genetic identity ($\bar{I}$) of Nei indicates a clear separation of E. sylvicola and E. torrentium on the one hand and E. alpicola and E. yougoslavicus on the other. But we can not estimate yet whether an index of 0.33 will clearly mark the genus level in Ephemeroptera, too. It will only be possible after further investigations (worldwide) of other Heptageniidae to verify wether all Epeorus-species of Tshernova belong to one genus.

Our results with the genus Epeorus show a good correlation of the data received with different methods. The proof for the usefulness of the biochemical method gives us the tool

for future differentiation of more difficult genera, where morphological characters are doubtful.

REFERENCES

Ayala, F.J., Powell, J.R., Tracey, M.L., Mourão, C.A. and Peres-Salas, S. 1972. Enzyme variability in the Drosophila willistoni group. IV. Genic variation in natural populations of Drosophila willistoni. Genetics, 70: 113 - 139.

Berthelemy, C. and Thomas, A. 1967. Note taxonomique sur Epeorus torrentium Eaton, 1881 et E. assimilis Eaton, 1885 (Ephemeroptera, Heptageniidae). Ann. Limnol., 3: 65 - 74.

Braasch, D. 1980. Iron yougoslavicus Šámal neu für Italien und Bulgarien (Insecta, Ephemeroptera, Heptageniidae). Faunist. Abh. (Dresden), 8: 81.

Clayton, J.W. and Tretiak, D.N. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. J. Fish. Res. Bd. Canada, 29: 1169 - 1172.

Ferguson, A. 1980. Biochemical systematics and evolution. Blackie, Glasgow/London.

Nei, M. 1971. Interspecific gene differences and evolutionary time estimated from electrophoretic data on protein identity. American Naturalist, 105: 385 - 398.

Puthz, V. 1978. Ephemeroptera. In: Illies, J. (Hrsg.): Limnofauna Europaea. Fisher, Stuttgart/New York.

Scholl, A., Corzillius, B. und Villwock, W. 1978. Beitrag zur Verwandschaftsanalyse altweltlicher Zahnkarpfen der Tribus Aphaniini (Pisces, Cyprinodontidae) mit Hilfe elektrophoretischer Untersuchungsmethoden, Z. zool. Syst. Evolut.-forsch., 16: 116 - 132.

Tomka, I. und Hasler, W. 1978. Einsatz des Rasterelektronenmikroskop bei taxonomischen Studien an Eintagsfliegen (Ephemeroptera). Bull. Soc. Frib. Sc. Nat., 67: 144 - 151.

Tshernova, O.A. 1981. On the systematics of adult mayflies of the genus Epeorus Eaton, 1881 (Ephemeroptera, Heptageniidae). Rev. Entomol. URSS, 60: 323 - 336 (in Russian).

Zurwerra, A. und Tomka, I. 1982. Enzymelektrophoretische Untersuchungen an der Gattung Epeorus Eaton (Heptageniidae, Ephemeroptera). Mitt. Schweiz. Entomol. Ges., 55: 359 - 360.