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Biochemical systematics and evolution of the European Heptageniidae (Ephemeroptera)

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With 3 figures and 7 tables in the text

Abstract

The phylogenetic relationships of 55 taxa of European Heptageniidae were investigated by using biochemical and morphological methods. To estimate genetic identity values the expression of sixteen gene loci was studied by analysing the electrophoretic mobilities of their products. The resulting pair-correlations generally confirmed relationships based on morphological data. The following systematic changes were established: 1) *Heptagenia joernensis* BGTSS., 1909, was placed in the nearctic genus *Nixe* Flowers, 1980. — 2) All representatives of the *Ecdyonurus lateralis* group were assigned to the genus *Electrogena* Zurwerra & Tomka, 1985. — 3) All *Rhithrogena* species, until recently divided into seven groups by Sowa, were assigned to two major groups, the *R. laevigata*-group and the *R. lobata*-group. — 4) *Rhithrogena ferruginea* Navás, 1905, *R. iridina* (Kolenati, 1860) and *R. iridina picteti* (Sowa, 1971) showed only small difference ($0.90 < \bar{I} > 0.97$) from *R. semicolorata* and were therefore considered to be subspecies of *R. semicolorata* (Curtis, 1834). — 5) Six new species were found: *Ecdyonurus alpinus* (Hefti/Tomka/Zurwerra, 1987) and *E. parahelveticus* (Hefti/Tomka/Zurwerra, 1986), *Electrogena hellenica, E. pseudograndiae* and *E. vipavensis* (all Zurwerra & Tomka, 1986) as well as *Rhithrogena intermedia* Metzler/Tomka/Zurwerra, 1987.

Introduction

A comprehensive revision of the systematics of the European Heptageniidae does not exist. Various authors have investigated single species (Metzler et al., 1985; Thomas & Sartori, 1985), species groups (Kimmins, 1958; Bogoescu & Tabacaru, 1962; Belfiore, 1981; Jacob, 1984) or genera (Jacob, 1974; Sowa, 1984). The first edition of the Limnofauna Europaea in 1968 (Illies, 1968) included 55 species subdivided into six genera. Seventy-five species are listed in the second edition (1978). This includes the genera *Epeorus, Epeiron, Rhithrogena, Ecdyonurus* and *Heptagenia*. A survey of the most recent literature (Tshernova, 1974; Belfiore, 1981, 1983; Braasch, 1983, 1984; Jacob & Braasch, 1984; Sowa, 1981, 1984; Sowa & Soldán, 1984 b; Zurwerra & Tomka, 1985) shows that today eight (Arthroplea genera *Arthroplea, Cinygma, Ecdyonurus, Electrogena, Epeorus, Heptagenia, Iron* and *Rhithrogena*) with a total of eighty-nine species are valid.

Various authors (Berlocher, 1984; Buth, 1984; Ferguson, 1980) have referred to the importance and the potential of enzyme electrophoresis as a tool

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to elucidate questions concerning systematics and phylogeny. The application of enzyme electrophoresis is especially useful when classical methods lead to unsatisfactory results. Encouraged by our own electrophoretic and morphological investigations on the genus *Epeorus* (ZURWERRA et al., 1984, 1986), in which biochemical and morphological data were highly correlated, we investigated all available European representatives of the Heptageniidae with the same standard methods. Special attention was paid to:

1) the status of the "*Ecdyonurus*" *lateralis*-group,

2) the status of *Ecdyonurus zelleri* within the *E. helveticus*-complex,

3) the status of "*joernensis*" within the genus *Heptagenia*,

4) the clarification of the *Rhithrogena semicolorata*-complex,

5) the subdivision of *Rhithrogena* into groups as proposed by JACOB (1974) and SOWA (1984).

The aim of the present work was to establish the relationships of fifty-five taxa of Heptageniidae analysed by biochemical means and to compare these results with findings based on morphological data. We made use of the two complementary methods in the following way: in case of a low correlations between the two approaches, we searched for new morphological features. This was usually successful. The results of the morphological investigations are included in this paper only if they are necessary to interpret systematic relationships.

The interpretation of the data from enzyme electrophoresis necessitates a precise definition of the term species. We follow the biological species concept of MAYR (1982): "Species are groups of interbreeding natural populations that are reproductively isolated from other such groups". The maintenance of a common gene pool among individuals of one species as a consequence of interbreeding, and the maintenance of reproduction barriers to exclude other species are of fundamental importance. The biological species concept can be applied only for geographically and chronographically coexistent populations, because of the limitation in time and space of species. These difficulties are omitted in the evolutionary species concept of SIMPSON, which takes into consideration the dimensions of space and time. For the interpretation of our data, however, the definition of MAYR is satisfactory.

The relationships between families of Ephemeroptera (EDMUNDS, 1962; KOSS, 1973; LANDA, 1973; RIEK, 1973) are usually discussed in terms of phylogeny and evolution. In discussing phylogenetic relationships within the family Heptageniidae, JENSEN & EDMUNDS (1970) divided the fifteen genera in their study into three phylogenetic lines, which are represented in Europe with (a) *Heptagenia* and *Ecdyonurus* (*Electrogena* included as "*lateralis*"-group), (b) *Rhithrogena* and *Cinygma* and (c) *Epeorus*. JACOB (1984) recognised three other European lines, (a) *Cinygma*, (b) *Epeorus* and *Rhithrogena*, (c) *Ecdyonurus* and *Heptagenia*. These assignments will be discussed later.

Table 1. List of taxa investigated with their countries of origin for material, and the number of animals and populations [for nomenclature, see PUTHZ (1978)]. Taxonomic determinations are based on morphological features and biochemical data.

Taxa	Number of animals	Number of populations	Origin*
Epeorus alpicola (ETN.)	45	3	CH
Epeorus sylvicola (PICT.)	144	7	CH, D, I, R
Epeorus torrentium ETN.	8	1	F
Epeorus yougoslavicus (ŠAMAL)	23	2	I
Rhithrogena alpestris ETN.	28	3	CH
Rhithrogena braaschi JACOB	6	1	GR
Rhithrogena colmarsensis SOWA	5	1	F
Rhithrogena degrangei SOWA	10	1	CH
Rhithrogena diaphana NAV.	19	2	CH, F
Rhithrogena endenensis ME/TO/ZU	25	2	CH
Rhithrogena germanica ETN.	8	1	CH
Rhithrogena hercynia LANDA	38	3	CH, I
Rhithrogena hybrida ETN.	47	4	CH, F
Rhithrogena insularis ESB.-PET.	8	1	F (Corsica)
Rhithrogena intermedia ME/TO/ZU	10	1	CH
Rhithrogena loyolaea NAV.	36	3	CH, YU
Rhithrogena nivata ETN.	20	2	CH
Rhithrogena puthzi SOWA	13	1	A
Rhithrogena semicolorata ferruginea[1]	24	3	CH, F, I
Rhithrogena semicolorata iridina[2]	19	2	R
Rhithrogena semicolorata picteti[3]	38	5	CH, D, F
Rhithrogena semicolorata semicolorata[4]	54	4	CH, D
Rhithrogena sibillina ME/TO/ZU	15	2	I
Ecdyonurus alpinus HE/TO/ZU	23	2	CH
Ecdyonurus angelieri THOMAS	2	1	F
Ecdyonurus aurantiacus (BURM.)	3	1	F
Ecdyonurus bellieri (HAG.)	12	2	F (Corsica)
Ecdyonurus carpathicus SOWA	10	2	R, YU
Ecdyonurus dispar (CURT.)	20	2	CH, F
Ecdyonurus forcipula (PICT.)	19	3	CH, F
Ecdyonurus helveticus (ETN.)	54	8	CH, F, I
Ecdyonurus insignis (ETN.)	22	1	D
Ecdyonurus krueperi (STEIN)	14	1	GR
Ecdyonurus macani THOMAS & SOWA	34	1	I
Ecdyonurus parahelveticus HE/TO/ZU	30	2	CH, F
Ecdyonurus picteti (MEYER-DÜR)	69	6	A, CH, F, YU
Ecdyonurus ruffii GRANDI	7	1	F
Ecdyonurus starmachi SOWA	14	3	H, R
Ecdyonurus subalpinus KLAP.	5	1	R
Ecdyonurus torrentis KIMM.	29	4	CH, D, GR, YU
Ecdyonurus venosus (F.)	82	6	CH, D, YU
Ecdyonurus zelleri (ETN.)	21	2	A, YU
Electrogena grandiae (BELFIORE)	15	1	I

Table 1. continued.

Taxa	Number of animals	Number of populations	Origin*
Electrogena gridellii (GRANDI)	13	2	CH, YU
Electrogena hellenica ZU/TO	17	1	GR
Electrogena lateralis (CURT.)	30	3	CH, YU
Electrogena ozrensis (TANASIJEVIĆ)	3	1	YU (Ozren)
Electrogena pseudograndiae ZU/TO	16	1	F (Corsica)
Electrogena quadrilineata (LANDA)	5	1	D
Electrogena vipavensis ZU/TO	4	1	YU
Heptagenia coerulans ROST.	7	1	F
Heptagenia dalecarlica BGTSS.	13	2	N
Heptagenia flava ROST.	2	1	F
Heptagenia sulphurea (MÜLL.)	30	5	CH, D, F, N
Nixe joernensis (BGTSS.)[5]	17	2	N

* A = Austria; CH = Switzerland; D = Federal Republic of Germany; F = France; I = Italy; GR = Greece; H = Hungary; N = Norway; R = Romania; YU = Yugoslavia. [1], subsp. n., syn. *Rhithrogena ferruginea* NAV.; [2], subsp. n., syn. *R. iridina* (KOL.); [3], comb. n., syn. *R. iridina picteti* SOWA; [4], subsp. n., syn. *R. semicolorata* (CURT.); [5], comb. n., syn., *Heptagenia joernensis* BGTSS.

Material and methods

Biochemical methods were used to clarify the relationships between the European Heptageniidae taxa (excluding Caucasian and Iberian species). We analysed the imagines of 124 populations by determining the electrophoretic mobilities of sixteen enzymes. In Table 1 all taxa investigated biochemically are listed together with their country of origin and the number of animals and populations studied. A population is represented by the average of ten imagines per taxon. The material to be investigated by electrophoresis was kept at −70 °C. No material was available for electrophoretic investigations of thirty-four species, of which many are endemic. During the time of sampling, often only larvae were available. Therefore, the majority of these had to be reared to the adult stage in the laboratory. In this way, all postembryonic developmental stages could be used for the morphological determinations. Ambiguous determinations were compared with holotypes or with locotypical material. Colour patterns were either observed on living mayflies or were registered photographically. For the electrophoretic investigation, we used only the imaginal stage. Prior to freezing, parts carrying important morphological characters (i.e. wings, legs, and the last two or three abdominal segments) were detached from these samples and stored in 70 % alcohol. The detached parts were then used for the morphological determinations.

Single individuals were homogenized in ten volumes of 0.1 M Tris-HCl buffer (pH 8.0) and centrifuged for 10 min at 8,000 g. Supernatant fractions of the individual samples were applied to starch gels containing 20 slots. The charged molecules were separated according to their different mobilities in an electrical field of 12 V/cm. Then the gels were cut in such a manner that in the resulting sublayers the enzymes of all the applied samples could be reacted with a specific substrate. For the visual observation of the position of the enzymes, a second reaction was necessary. The enzyme sub-

strate stainings were performed according to standard methods (BREWER, 1970; AYALA et al., 1972; SCHOLL et al., 1978).

Sixteen enzymes were analysed: Adenylate kinase (* AK), Arginine kinase (* APK), Aldolase (** ALD), Glutamate-oxaloacetate transaminase (** GOT-1 and ** GOT-2), α-Glycerophosphate dehydrogenase (** α-GPDH), Indophenol oxidase (** IPO-1 and ** IPO-2), Hexokinase (** HK-1 and ** HK-2), Leucine aminopeptidase (*** LAP), Malate dehydrogenase (** MDH-1 and ** MDH-2), Mannose phosphate isomerase (*** MPI), Phosphoglucomutase (** PGM) and Retinol dehydrogenase (*** RDH). The following buffers were used for enzymatic reactions: * N-(3-Aminopropyl)-morpholine-citrate, pH 6.0; ** N-(3-Aminopropyl)-morpholine-citrate, pH 7.0; *** Tris-borate-EDTA buffer, pH 9.0 (for details, see ZURWERRA et al., 1986).

The genetic interpretation of phenotypes as detected by enzyme analysis could not be tested by crossbreeding experiments. Therefore, the various electromorphs were interpreted on the basis of their analogy to other enzymatically investigated groups (GEIGER, 1980; SCHOLL et al., 1980). The allelic variation of a given enzyme was expressed by comparing the position of the electromorphs of a particular sample with that of the most frequent electromorph of the corresponding enzyme in *Epeorus sylvicola*, for which the index 100 was chosen arbitrarily.

In contrast to the conventional method of evaluation (AYALA et al., 1972; GEIGER, 1980), the relative mobility index ($RMI_{x,i}$) was defined for the electromorph of the population i (subscript i) as follows:

$$RMI_{x,i} = \frac{(m_{x,i} - m_{x,ref})}{m_{x,ref}} \times 10 + 100$$

where $m_{x,i}$ and $m_{x,ref}$ are the distances of the positions (measured in mm from the gel origin) of the sample and reference electromorphs of the same electrophoretic run (ZURWERRA et al., 1986). Use of this formula reduced the source of data scatter inherent to the electrophoretic separation on starch gels. In cases where sample and reference electromorphs migrate very close to each other, there was a loss of information as a consequence of rounding-up rules in this formula. This effect, which normally reduced the scattering of the mobility of well separated electromorphs, could also suppress effective differences. When the reproducibility was sufficient, half integers were introduced. Electromorphs designated by half integers were also used for the calculation of genetic identity values. The relative mobility indices could be compared for pairs of taxa (correlation analysis; NEI, 1972) and are presented graphically in the form of a dendrogram according to the unweighted pair-group arithmetic average clustering method (FERGUSON, 1980). The RMI-value between two taxa compared is a measure of the degree of genetic similarity and is therefore called genetic identity value (I-value).

Results

Table 2 lists the main electromorphs of all loci studied in order of increasing interspecific variability of each taxon. Relatively conservative enzymes for which only a few electromorphs were observed (e.g. only two electromorphs for MDH-2) are arranged on the left hand side, while the polymorphic ones containing many electromorphs (e.g. HK-2) are placed on the right hand side of Table 2. All electromorphs with their observed frequencies are listed for the Ald- and Mdh-1 loci in the *Rhithrogena semicolorata*-complex (Table 4) and for

Table 2. The main electromorphs at sixteen loci for all taxa investigated.

Enzymes / Taxa	MDH-2	GPDH	APK	RDH	MPI	ALD	HK-1	GOT-2	AK	IPO-1	IPO-2	PGM	GOT-1	LAP	MDH-1	HK-2
Ep. alpicola	100	100	100	99	100	99	101	102	105	100	96	97	96	99	98	103
Ep. sylvicola	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Ep. torrentium	100	100	100	100	100	98	100	100	100	100	100	100	99	100	99	100
Ep. yougoslavicus	100	100	100	99	100	99	104	102	105	100	96	102	105	98	100	113
R. alpestris	100	99	100	101	100	96	100	101	119	100	100	101	100	98	98	104
R. braaschi	100	98	98	100	100	96	103	101	115	99	96	101	106	98	96	109
R. colmarsensis	100	98	98	99	101	99	102	100	115	99	96	102	106	99	98	109
R. degrangei	100	99	98	100	99	99	100	101	119	100	103	102	107	100	95	109
R. diaphana	98	98	98	101	101	99	101	100	115	102	96	101	108	101	98	109
R. endenensis	100	99	98	101	99	99	102	101	119	100	104	104	106	98	95	109
R. germanica	100	98	98	101	99	96	104	99	115	99	96	102	106	101	98	115
R. hercynia	100	99	98	100	99	99	102	99	119	100	103	102	101	99	95	109
R. intermedia	98	99	98	100	99	99	100	100	115	100	100	101	104	101	98	109
R. hybrida	100	99	98	100	99	99	102	101	119	100	104	102	101	100	95	109
R. insularis	100	99	98	100	99	96	102	101	119	100	103	103	98	99	95	107
R. loyolaea	100	99	98	100	100	99	103	101	119	99	96	104	101	100	98	109
R. nivata	100	99	98	101	99	96	100	100	119	97	103	101	101	98	95	107
R. puthzi	100	99	98	101	99	96/98	102	101	119	100	104	104	106	100	95	109
R. semicolorata ferruginea	100	98	98	101	100	99	103	99	115	99	96	102	106	101	98	109
R. semicolorata iridina	100	98	98	101	100	100	103	99	115	99	96	102	106	101	98	109
R. semicolorata picteti	100	98	98	101	100	99	103	99	115	99	96	102	106	101	96	109
R. semicolorata semicolorata	100	98	98	101	100	99	103	99	115	99	96	102	106	101	96	109
R. sibillina	100	99	98	101	99	95	102	101	119	100	104	102	101	100	95	109
Ec. alpinus	100	100	98	101	99	95	102	99	106	96	97	101	105	98	100	108
Ec. angelieri	100	100	***	100	101	96	102	101	106	99	102	101	101	96	98	108
Ec. aurantiacus	100	100	100	101	101	96	102	98	106	99	102	103	101	**	98	108
Ec. bellieri	100	100	100	101	101	95	102	98	106	99	102	101	101	99	98	108
Ec. carpathicus	100	100	98	100	101	97	102	99	106	99	101	102	105	98	100	108

Table 2. continued.

Enzymes / Taxa	MDH-2	GPDH	APK	RDH	MPI	ALD	HK-1	GOT-2	AK	IPO-1	IPO-2	PGM	GOT-1	LAP	MDH-1	HK-2
Ec. dispar	100	100	100	100	102	98	102_5	98	106	99	102	100	105	96	98	110
Ec. forcipula	100	100	96	100	101	96	102	97	106	99	102	101	101	96	98	108
Ec. helveticus	100	100	98	101	100	95	102	101	106	96	97	101	105	97	100	108
Ec. insignis	100	100	100	101	101	96	102	100	106	101	102	102	101	97	98	108
Ec. krueperi	100	100	100	101	101	100	101	99	106	99	97	101	105	99	98	107
Ec. macani	100	100	98	100	101	96	102	97	106	99	102	101_5	101	98	100	108
Ec. parahelveticus	100	100	98	101	100	95	102	101	106	96	97	101	106	97	98	108
Ec. picteti	100	100	98	101	100	95	103	99	106	96	97	102	106	98	100	113
Ec. ruffii	100	100	100	100	102	96	102	98	106	99	102	101	101	96	100	108
Ec. starmachi	100	100	100	101	102	96	102	97	106	99	103	103	105	98	100	108
Ec. subalpinus	100	100	98	100	100	95	102	98	106	99	101	101	105	98	98	108
Ec. torrentis	100	100	98	100	101	96	102_5	99	106	99	102	101	105	98	95	110
Ec. venosus	100	100	96	100	101	96	102	97	106	99	102	102	101	97	98	108
Ec. zelleri	100	100	100	101	100	95	102	99	100	96	97	101	105	97	98	108
El. grandiae	98	98	100	101	102	96	103	99	101	99	101	99	108	100	103	110
El. gridellii	98	99	98	99	101	96	103	100	112	98	102	97	108	100	98	107
El. hellenica	98	99	100	99	101	95	101	99	107	101	101	97	108	100	98	102
El. lateralis	98	99	100	99	101	96	102	100	107	101	101	97	108	100	98	107
El. ozrensis	98	99	100	99	101	96	103	100	107	98	101	98	108	101	98	103
El. pseudograndiae	98	98	100	99	102	96	102	100	107	99	102	97	108	100	101	107
El. quadrilineata	98	99	100	99	101	96	103	100	112	98	102	97	108	100	98	108
El. vipavensis	98	99	100	99	101	96	103	99	107	98	101	97	108	100	98	103
H. coerulans	98	100	99	98	101	99	100	100	112	98	96	99	108	101	94	102
H. dalecarlica	98	100	99	99	101	98	100	98	112	99	96	102	108	98	96	102
H. flava	98	100	99	99	101	99	99	98	107	98	96	101	108	102	94	102
H. sulphurea	98	100	99	98	101	98	99	97	112	98	96	101	108	101	97	101
N. joernensis	98	99	100	98	100_5	95	103	99	106	96	96	102	104	97	100	113

the Got-1-locus in different populations of *Ecdyonurus venosus* (Table 6). The intraspecific frequency differences for this taxa are discussed. For the calculation of the Ī-values however, all electromorphs were considered. Table 7 gives the Ī-values calculated for pairs of taxa. These values were used to generate the dendrogram (Fig. 1). The biochemical data which are relevant for the systematics of the Heptageniidae are summarized in Fig. 2.

Presentation of the taxa

Specific electromorphs or pairs of electromorphs were found for the systematic ranks of genus, subgenus (species-group) and species (see AK, APK, GOT-2, α-GPDH etc., in Table 2). Some of the allelic variants were common to more than one genus (see electromorphs 98 and 100 at the Mdh-2 locus).

Epeorus

Recent data from analytical enzyme electrophoresis (Zurwerra et al., 1986) have shown that the four European species available to us (see Table 1; *E. zajtzevi* could not be investigated) can be clearly subdivided into two groups. One group contains the taxa *E. alpicola* and *E. yougoslavicus* which were regarded by some authors as being members of the genus *Iron* Eaton, 1883. Our findings indicate no close relationship between the type species of *Iron* (*I. longimanus*, North America) and *E. alpicola* — *E. yougoslavicus*. The calculated low genetic identity values between 0.19 and 0.21 (see Zurwerra et al., 1986) correspond to the genus level within the Heptageniidae. Common electromorphs between *I. longimanus* and the European *Epeorus* taxa were only found for α-GPDH, IPO-2 and MDH-1 among a total of sixteen enzymes investigated. The four European taxa of *Epeorus* can be subdivided into the *E. sylvicola*- and *E. alpicola*-group, which are characterized by the AK, GOT-2, IPO-2 and RDH enzymes (see Table 2).

Later morphological comparisons (Tomka & Zurwerra, 1985) of *E. alpicola* and *E. yougoslavicus* with the nearctic representatives of the subgenus *Ironopsis* Traver, 1935 (sensu Tomka & Zurwerra, 1986) and of the genus *Epeorus* showed that both European species fulfill all the criteria of the nearctic subgenus *Ironopsis*. The genetic identity coefficient of 0.35 between the *E. alpicola*- and *E. sylvicola*-group also supports the subgeneric rank of both groups, since this value is distinctly lower than those between any congeneric species-groups of the Heptageniidae (see Fig. 1).

Rhithrogena

Jacob (1974) and Sowa (1984) divided the numerous species of *Rhithrogena* (more than ⅓ of the European Heptageniidae belong to *Rhithrogena*) in groups

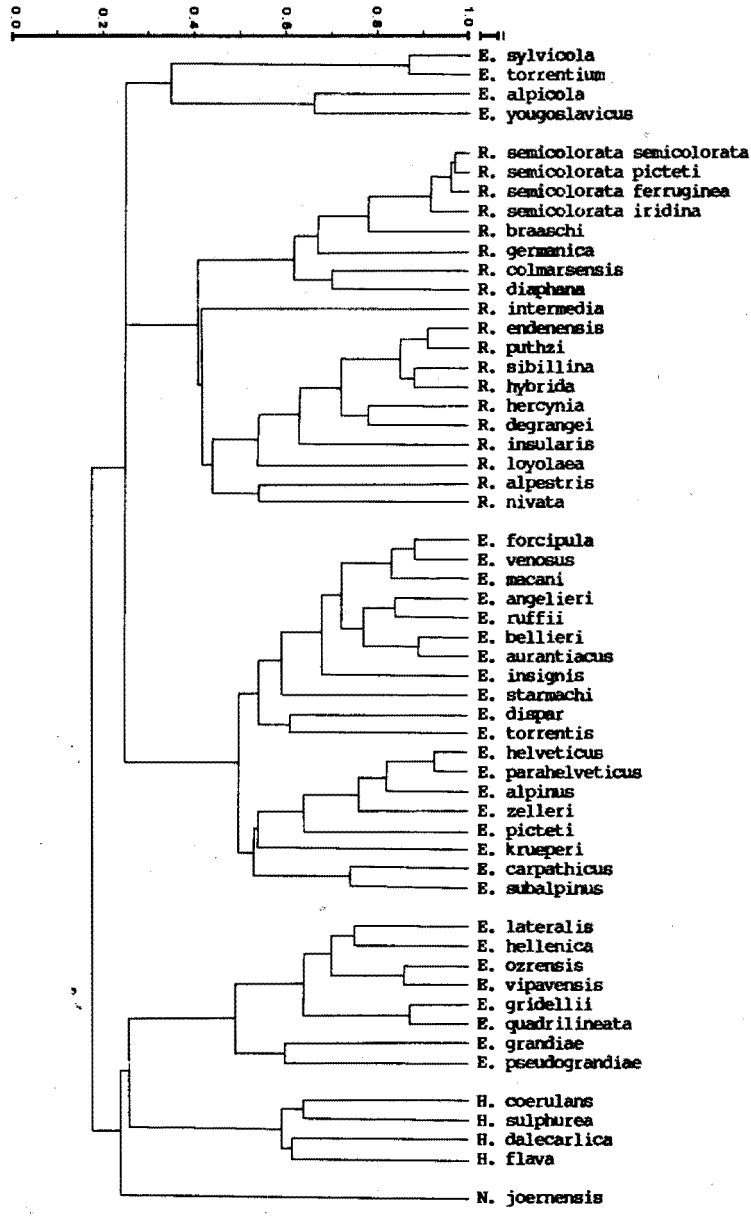

Fig. 1. Dendrogram of the fifty-five taxa investigated.

for a better understanding of their interrelationships. Both authors used larval and imaginal characteristics and established six and seven groups respectively. The investigation of these groups with biochemical methods showed a remarkable result. Instead of separating into many minor groups, all *Rhithrogena*

species investigated were clustered into two distinct major groups (Figs. 1, 2) which are defined by the diagnostic characteristics given in Table 3 and Fig. 3.

Examination of Table 2 with respect to the major groups of *Rhithrogena* shows, besides α-GPDH, AK as a second diagnostic enzyme with the exception of *R. intermedia* METZLER/TOMKA/ZURWERRA, 1987. This new species was the only member of the *R. lobata*-group with electromorph 115 of AK, a con-

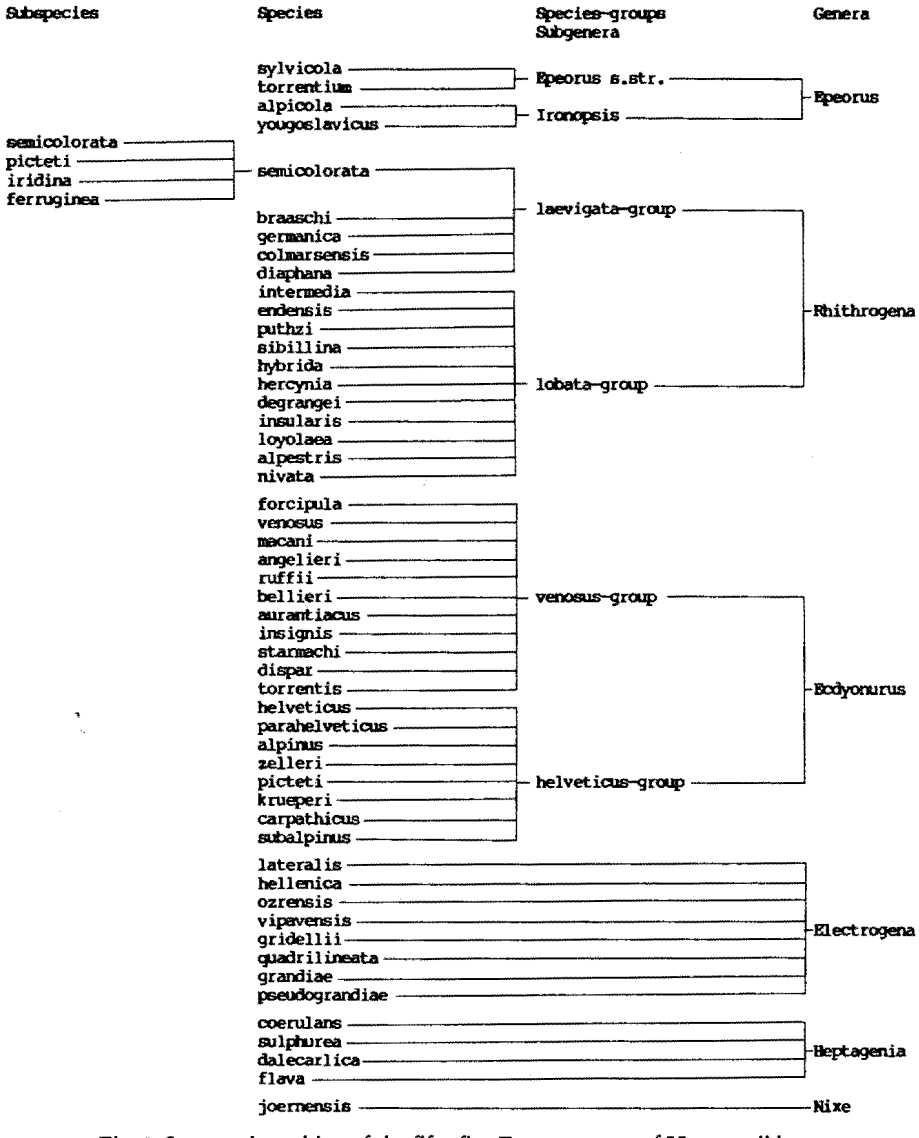

Fig. 2. Systematic ranking of the fifty-five European taxa of Heptageniidae.

Table 3. Characteristic of the *Rhithrogena laevigata-* and *R. lobata*-group.

	laevigata-group	*lobata*-group
larval characteristics:		
edge of gills	edge of 7th gill smooth (BELFIORE, 1983: 75, Fig. 6a–g; SOWA, 1970: Figs. 28–30)	edge of all gills lobed (SOWA, 1984: Figs. 29–32)
front edge of the lateral sclerite on the 1st abdominal sternite	is turned backwards (SOWA, 1970: 77, Fig. 25)	is turned forwards (SOWA, 1984: 39, Fig. 2)
imaginal characteristics:		
form of the apical edge of the ejaculatory duct of the penis-lobes	the protruding apical edge is at the same level (Fig. 3a) and surrounds the funnel-shaped ejaculatory duct. The sloping of the funnel-shaped edge is steady all-around	the protruding apical edge is not placed at the same level. It is only visible on the ventral side of the often rift-shaped orifices of the ejaculatory duct; the hemispherical dorsal edge slopes gradually to the ventral part of the orifice (Fig. 3b)
α-Glycerophosphate dehydrogenase	electromorph 98	electromorph 99

dition which in general was valid for the *R. laevigata*-group. It has to be emphasized that in two out of ten individuals studied, the electromorph 119 occurred in addition to the main electromorph 115. The Ī-value was only by an average of 0.05 units higher in comparison with the representatives of the *R. lobata*-group than with species of the *R. laevigata*-group. On the other hand, *R. intermedia* had the highest Ī-value when compared to *R. diaphana* (Ī = 0.62), which belongs to the *R. laevigata*-group. The intermediary status of *R. intermedia* within all *Rhithrogena* species was documented by the biochemical features, e.g. the electromorph 98 at the Mdh-2-locus occurred only in *R. diaphana* and *R. intermedia* among all *Rhithrogena* studied. Furthermore, the absence of the plica on the lamella of the first gill is a common feature of *R. intermedia, R. alpestris, R. diaphana* and *R. eatoni*. Despite similarities with *R. diaphana, R. intermedia* was placed in the *R. lobata*-group (see Table 3).

SOWA (1984) recognised thirty-six *Rhithrogena* species, of which twenty-seven are valid today, and subdivided them into seven minor groups. In the present work, sixteen out of these taxa were considered and complemented with the recently identified new species *R. endenensis, R. sibillina* (METZLER et al., 1985) and *R. intermedia* (METZLER et al., in preparation). The assignment to

the major groups can be seen in Figs. 1 and 2. The remaining species placed in the *R. alpestris*-, *R. hybrida*- and *R. loyolaea*-group by Sowa could be assigned to the *R. lobata*-group with the exception of *R. gorganica*, which belongs to the *R. laevigata*-group. In addition all other known species of the *R. semicolorata*-, *R. germanica*-, *R. sowai*- and *R. diaphana*-group that were not investigated by biochemical means could be assigned to the *R. laevigata*-group, with the exception of *R. buresi*, *R. eatoni* and *R. sowai*. These clearly belong to the *R. lobata*-group. *R. datterai* is synonymous with *R. castellana* (cf. Thomas & Sartori, 1985) and belongs also to the *R. laevigata*-group. According to our results, the shape of the seventh gill is the most important criterion to differentiate the major groups. Sowa disregarded this criterion and placed *R. eatoni* with lobed and *R. gorganica* with smooth edged gills into the wrong minor groups. (We found the larval stage of *R. eatoni* for the first time on Corsica.)

The results of enzyme electrophoretic analyses on representatives of the *Rhithrogena semicolorata*-group were astonishing. The four taxa *R. semicolorata iridina* comb. n., *R. semicolorata picteti* comb. n., *R. semicolorata ferruginea* comb. n. and *R. semicolorata semicolorata* subsp. n. could not be differentiated by the enzymatic criteria (see Table 2, 7; Fig. 2), although Sowa was able to discriminate them by means of penis morphology and wing-colouration of imagines (the larvae are not discernible). The comparison of these taxa with the main electromorphs (Table 2) showed identity for all enzymes investigated, except for ALD and MDH-1. The analysis of all electromorphs observed for these two enzymes (Table 4) showed that the variation of the genetic identity values (0.90—0.97, see Table 7) are the result of frequency differences. Are these high Ī-values already sufficient for the species criterion? According to the definition of a biological species by Mayr (see introduction) the presence of a common gene pool is a basic requirement. The comparison of the four taxa (Table 4) showed that these are not reproductively isolated. Gene flow is supposed to occur because the investigation of a total of 135 imagines representing fourteen geographically isolated populations (Table 1) did not show significant differences in the frequencies of the MDH-1 electromorphs. On the contrary, the frequency differences of ALD slightly indicated reproductive isolation.

Table 4. The frequencies of electromorphs at the Ald- and Mdh-1 loci of four taxa of the *Rhithrogena semicolorata*-complex. The number of populations investigated is given in parentheses.

Electromorph	ALD		MDH-1	
	99	100	96	98
R. semicolorata ferruginea (3)	81	19	49	51
R. semicolorata iridina (2)	–	100	36	64
R. semicolorata picteti (5)	92	8	62	38
R. semicolorata semicolorata (4)	91	9	54	46

The presence of electromorph 100 as the only electromorph in both *R. semicolorata iridina* populations, is by itself not sufficient since some populations of *R. semicolorata semicolorata* and *R. semicolorata picteti* also had only the electromorph 99. We propose that *R. ferruginea*, *R. iridina* and *R. iridina picteti* (Sowa, 1970) should be regarded as subspecies of *R. semicolorata* (Fig. 2) because the four taxa share a common gene pool with identical allelic variants. The differences of the penis structures used by Sowa (1970) were reinvestigated by us on more extensive material. The morphological characteristics do not allow to separate *R. semicolorata ferruginea*, *R. semicolorata iridina* and *R. semicolorata picteti*. These taxa can be identified only by the colouration of the wings. Only in some exceptional cases, is it possible to separate *R. semicolorata semicolorata* from the three others by morphological features.

Both newly described species, *R. endenensis* and *R. sibillina* (Metzler et al., 1985) can also be distinguished biochemically (by a combination of allelic variants) from all other *Rhithrogena* species. With an Ī-value of 0.91, *R. endenensis* showed a strong affiliation to *R. puthzi*. Both species can be distinguished at the Ald- and Lap-loci (see Table 2). *R. puthzi* showed the electromorph 98 in addition to the electromorph 96. The former did not occur in the other *Rhithrogena* species. *R. sibillina*, which can be determined rather easily by morphological criteria, showed a close relationship to *R. hybrida*, *R. puthzi* and *R. endenensis*, but differed from them at the Ald-locus, from *R. puthzi* and *R. endenensis* also at the Pgm- and Got-1-locus (see Table 2).

Ecdyonurus

Prior to the separation of the "*lateralis*"-group (defined as a new genus *Electrogena* Zurwerra & Tomka, 1985) from the genus *Ecdyonurus*, the latter contained thirty-six species in Europe (without the Caucasian ones). The now remaining twenty-three species, discernible by larval and imaginal characteristics, can be assigned to two groups (Table 5).

Twenty-four populations of the *E. helveticus*-group were analysed electrophoretically. After inspection of the biochemical data, individuals of four populations, identified as *E. helveticus* by the morphological predetermination, could not be assigned to a known species. The presence of specific allelic variants defined these populations as two new species, namely *E. alpinus* Hefti/Tomka/Zurwerra, 1987 and *E. parahelveticus* Hefti/Tomka/Zurwerra (Hefti et al., 1986, 1987). The highest genetic identity was found between *E. parahelveticus* and *E. helveticus* with an Ī-value of 0.93. However, both species are easily distinguishable at the Got-1-locus. The populations of *E. parahelveticus* from the Alps of Valais and Savoie (Table 1) showed the same electromorphs (main electromorph 106, adjacent one 108), which were usually typical for *E. picteti* within the *E. helveticus*-group. The electromorph 106 was charac-

Table 5. Characteristics of the *Ecdyonurus helveticus*- and *E. venosus*-group.

	helveticus-group	*venosus*-group
larval characteristics:		
pilosity on hypopharynx	few hairs on lateral lobes	dense hairs on lateral lobes
hind projections on abdominal tergites	short projections	long projections
spines near the distal part of hind femur	spatulated or rounded apically	lancelolate, sharp
imaginal characteristics:		
penis-lobes	along the curvature of penis-lobes with apical elongated inner sclerite (apical sclerite) (KIMMINS, 1958: 230, Fig. 12)	inner sclerite not elongated apically; the distal part often overlooks the median edge of penis-lobes (THOMAS, 1970: 78, Fig. 8)
Indophenol oxidase-2	electromorph 97 or 101	electromorph 102 or 103

terized by the presence of a coloured dilatation directed to the anode (probably active cleavage products), and can be easily distinguished from the electromorph 105 of all the other representatives of the *E. helveticus*-group (Table 2). With the exception of the electromorph 98, which replaces the electromorph 100 at the Mdh-1-locus, the remaining biochemical characteristics are identical to those of *E. helveticus*. The two different main electromorphs result from a frequency difference at this locus. The male imagines of *E. parahelveticus* can be distinguished from all other members of the group by the length to width ratio <2 of the lateral sclerite of the penis-lobes. In contrast to all known species, larval diagnostic features are the short and not densely distributed projections on the hind border of the abdominal tergites and the missing teeth on the tarsal claws. Full details with respect to the diagnostic differences of *E. parahelveticus* are given by HEFTI et al. (1986).

Despite the high similarity regarding these characteristics, we detected distinct differences between the two alpine populations of Lochbach and Ova dal Crot of Switzerland and *E. helveticus* populations. At the Got-2-locus a 100% difference was observed between the electromorphs of *E. helveticus* and both alpine populations. In the alpine populations, the enzyme GOT-2 was presented by electromorph 99, which also occurred in all populations of *E. carpathicus, E. krueperi, E. zelleri* and again *E. picteti* (Table 2), whereas in *E. helveticus* and *E. parahelveticus* it was represented by the electromorph 101. This allelic difference and other characteristics allowed the identification of these populations as distinct species. According to MAYR's definition of a species,

this population has been described as a distinct taxon, *E. alpinus* (Hefti et al., 1987). The name indicates the ecological distribution.

Both species, *E. alpinus* and *E. parahelveticus*, were closely related to *E. helveticus* and therefore similar to members of the *E. helveticus*-complex sensu Kimmins (1958), which included until now the three species *E. helveticus*, *E. austriacus*, *E. zelleri*, which are all distinguishable by penis morphology and patterns on subimaginal wings. In 1975 Puthz abrogated the name *E. austriacus* and declared it as a synonym of *E. picteti*. This interconvertion was mainly based on subimaginal wing patterns, which, indeed, are very similar in both species.[1] According to the descriptions of Kimmins (1958), *E. austriacus* shows clearly zig-zag patterns, *E. helveticus* diffuse striped patterns and *E. zelleri* uniformly-grey wings. Our verification of these patterns revealed local overlaps with more than one species of the *E. helveticus*-complex. For example in Radovna (YU), we expected to find the three species *E. helveticus*, *E. picteti* and *E. zelleri* according to the predeterminations. However, using the enzyme electrophoretic data and morphological features, only the species *E. picteti* and *E. zelleri* could be identified. The clearly visible zig-zag pattern on the subimago wings, allowed to separate *E. picteti* (and also *E. austriacus*) from the other species of the *E. helveticus*-complex. The result from this analysis showed that, *E. helveticus* exhibits patterns which vary from diffused zig-zag to uniformly-grey. The same phenomenon was also observed in *E. alpinus* and *E. parahelveticus*. The difficulty of the interpretation between diffused zig-zag or uniformly-grey wings of the subimago must also have led to the ambiguous species status of *E. zelleri*. This problem was obvious from the beginning, especially with regard to the populations of *E. helveticus* with uniformly-grey wings. The status of the *E. helveticus*-complex could be clarified using biochemical methods: *E. zelleri* was clearly separated from the other representatives of the *E. helveticus*-group by having the electromorphs 100 of AK and APK (Table 2).

The intermediary status of *E. picteti* among the species distributed in the Alps (*E. alpinus*, *E. helveticus*, *E. parahelveticus* and *E. zelleri*) and the East European species *E. carpathicus*, *E. krueperi* and *E. subalpinus* is clearly visible in the dendrogram and has been established by analysis the relative mobility of the sixteen enzymes studied (Fig. 1). The comparative Ī-values of *E. picteti* and the alpine species are betwen 0.60 and 0.69 (Table 7). The genetic identity decreased to a value of 0.48 between *E. picteti* and the East European species. A high Ī-value has been found only between *E. carpathicus* and *E. subalpinus* (both occur together in the Carpathians, see Limnofauna Europaea). No particular

[1] Recent investigations of the "Ephemeroptera working group" of Freiburg/Switzerland show that *E. austriacus* Kimmins, 1958, is a species propria (details see Hefti & Tomka, 1986).

similarity could be established between *E. krueperi* and members belonging to the *E. helveticus*-group ($0.48 < \bar{I} > 0.63$). However, a relatively high biochemical relatedness was found between *E. subalpinus* and members of the *E. helveticus*-complex, with the exception of *E. picteti*. The two species *E. epeorides* and *E. siveci* of the *E. helveticus*-group are not considered here (details see JACOB & BRAASCH, 1984).

The relationships based upon biochemical data are represented in Fig. 1 for the taxa belonging to the *E. venosus*-group. The assignment of some populations to known species like *E. angelieri*, *E. forcipula*, *E. ruffii* and *E. venosus*, however, was very difficult, although these could be clearly discriminated according to the calculated identity values. On the one hand, the difficulties resulted from a lack of exact morphological descriptions, and on the other hand from variations within the described features. Although both species have been described very early, the morphological separation of *E. forcipula* PICTET, 1843, from *E. venosus* FABRICIUS, 1775, is one of the most difficult problems of the systematic classification of the Heptageniidae. The reexamination of the published morphological characteristics (KIMMINS, 1942; THOMAS, 1968) showed considerable overlaps, despite a distinct biochemical separation ($\bar{I} = 0.88$) and the presence of a 100% allelic difference at the Pgm-locus (Table 2). The distal edge of the lateral sclerite, which is round in *E. forcipula* but straight in *E. venosus*, was the best diagnostic feature. The larval (hind borders and lateral projections of abdominal tergites) and the remaining imaginal characteristics (ratio of tibia : tarsus, teeth on forceps-base, abdominal patterns) were not constant. Even a more extensive analysis did not yield reliable features. *E. venosus* generally shows a greater variability (especially in body size) and is more widely distributed in Europe than *E. forcipula*. Furthermore, intraspecific variability at the Got-1-locus was observed for *E. venosus* (Table 6). This enzyme allows the six populations studied to be assigned to either of two groups. The main electromorph was 105 in both populations of Argen (D) and Gotteron (CH). This electromorph is usually found in *E. dispar*, *E. starmachi* and *E. torrentis*, all belonging to the *E. venosus*-group. Our study did not allow a further

Table 6. The frequencies of electromorphs at the Got-1-locus of different *Ecdyonurus venosus* populations.

Populations	Electromorphs at Got-1-locus		
	101	105	108
Argen	7	86	7
Gotteron	9	91	–
Hongrin	83	17	–
Sense	100	–	–
Vipava	100	–	–
Wutach	73	27	–

Table 7. Estimates of genetic identity values Ī in fifty-five European taxa of Heptageniidae.

	alp	syl	tor	you	alp	bra	col	deg	dia	end	ger	her	hyb	ins	int	loy	niv	put	sem	sem	sem	sem	sib	alp	ang	aur	bel	car	dis	for
Ep. alpicola	–	.36	.33	.66	.30	.21	.31	.22	.23	.24	.19	.25	.24	.22	.13	.27	.09	.21	.27	.22	.28	.27	.15	.20	.26	.25	.28	.15	.26	.15
Ep. sylvicola		–	.87	.35	.49	.21	.21	.32	.17	.18	.09	.21	.25	.20	.34	.27	.22	.29	.17	.20	.17	.15	.21	.23	.30	.25	.22	.22	.34	.22
Ep. torrentium			–	.34	.45	.21	.19	.31	.15	.17	.08	.20	.24	.20	.34	.25	.22	.30	.15	.13	.14	.14	.20	.22	.29	.23	.22	.21	.36	.22
Ep. yougoslavicus				–	.31	.26	.29	.28	.18	.25	.27	.25	.30	.15	.12	.26	.12	.19	.32	.26	.33	.33	.20	.28	.22	.23	.20	.36	.29	.14
R. alpestris					–	.36	.10	.47	.26	.50	.23	.31	.42	.44	.37	.39	.54	.56	.24	.26	.24	.30	.49	.29	.36	.33	.29	.13	.23	.22
R. braaschi						–	.63	.38	.55	.41	.61	.31	.38	.32	.37	.62	.29	.46	.78	.77	.77	.80	.30	.25	.40	.24	.22	.35	.20	.35
R. colmarsensis							–	.38	.70	.45	.60	.46	.46	.27	.45	.50	.23	.40	.71	.66	.71	.72	.33	.23	.29	.31	.34	.41	.20	.33
R. degrangei								–	.29	.70	.28	.78	.82	.63	.56	.64	.51	.69	.34	.30	.34	.35	.69	.23	.22	.10	.10	.30	.12	.16
R. diaphana									–	.33	.53	.29	.26	.10	.62	.38	.35	.27	.66	.62	.65	.70	.27	.24	.21	.25	.26	.20	.14	.21
R. endenensis										–	.34	.68	.86	.59	.46	.61	.49	.91	.41	.39	.44	.47	.82	.37	.22	.24	.24	.27	.14	.18
R. germanica											–	.36	.29	.27	.27	.33	.34	.34	.71	.73	.73	.71	.33	.33	.22	.30	.25	.36	.20	.23
R. hercynia												–	.80	.67	.54	.61	.51	.64	.43	.35	.41	.40	.69	.33	.25	.24	.26	.39	.12	.27
R. hybrida													–	.62	.50	.69	.45	.85	.35	.31	.36	.36	.88	.30	.29	.22	.21	.34	.12	.25
R. insularis														–	.32	.43	.55	.64	.14	.16	.16	.15	.56	.23	.30	.27	.22	.27	.11	.26
R. intermedia															–	.44	.46	.40	.38	.30	.34	.38	.38	.19	.15	.06	.11	.15	.08	.17
R. loyolaea																–	.39	.65	.56	.50	.56	.57	.59	.23	.37	.26	.25	.29	.20	.32
R. nivata																	–	.29	.23	.23	.25	.29	.53	.35	.28	.33	.27	.21	.15	.27
R. puthzi																		–	.38	.40	.39	.40	.84	.35	.27	.26	.23	.26	.17	.22
R. semicolorata ferruginea																			–	.92	.96	.95	.32	.32	.18	.23	.22	.37	.20	.20
R. semicolorata iridina																				–	.92	.90	.35	.31	.18	.25	.23	.32	.21	.17
R. semicolorata picteti																					–	.97	.33	.33	.16	.25	.22	.33	.21	.17
R. semicolorata semicolorata																						–	.34	.36	.21	.27	.28	.35	.22	.22
R. sibillina																							–	.42	.25	.30	.34	.27	.13	.21
Ec. alpinus																								–	.41	.50	.55	.59	.40	.42
Ec. angelieri																									–	.81	.71	.54	.59	.85
Ec. aurantiacus																										–	.89	.54	.62	.76
Ec. bellieri																											–	.46	.57	.69
Ec. carpathicus																												–	.41	.57
Ec. dispar																													–	.49
Ec. forcipula																														–

Table 7. Continued.

	hel	ins	kru	mac	par	pic	ruf	sta	sub	tor	ven	zel	gra	gri	hel	lat	ozr	pse	qua	vip	coe	dal	fla	sul	joe
Ep. alpicola	.23	.25	.32	.17	.22	.25	.23	.22	.23	.17	.16	.30	.07	.16	.29	.21	.26	.20	.23	.30	.20	.20	.25	.15	.19
Ep. sylvicola	.24	.30	.29	.26	.22	.24	.31	.23	.30	.21	.23	.37	.13	.16	.17	.21	.16	.18	.23	.19	.19	.15	.07	.11	.10
Ep. torrentium	.22	.27	.22	.23	.21	.21	.29	.20	.28	.22	.21	.35	.13	.14	.14	.23	.13	.18	.20	.16	.19	.20	.08	.17	.08
Ep. yougoslavicus	.29	.30	.31	.20	.20	.40	.25	.34	.34	.26	.23	.37	.07	.08	.15	.13	.13	.13	.14	.13	.19	.32	.25	.17	.31
R. alpestris	.28	.30	.32	.19	.36	.25	.25	.32	.25	.26	.15	.33	.23	.18	.20	.25	.23	.17	.26	.25	.08	.11	.07	.07	.19
R. braaschi	.34	.17	.22	.39	.41	.38	.31	.25	.43	.44	.31	.18	.27	.21	.01	.07	.17	.20	.15	.16	.12	.24	.11	.14	.16
R. colmarsensis	.23	.34	.29	.41	.30	.28	.26	.22	.32	.28	.37	.16	.14	.27	.14	.25	.25	.30	.21	.23	.30	.30	.24	.18	.15
R. degrangei	.24	.17	.10	.25	.23	.23	.18	.18	.24	.24	.23	.23	.05	.20	.14	.14	.10	.06	.14	.14	.13	.13	.07	.03	.16
R. diaphana	.25	.28	.29	.22	.26	.22	.11	.15	.20	.23	.16	.16	.29	.33	.21	.26	.34	.27	.26	.26	.46	.34	.33	.33	.20
R. endenensis	.39	.23	.22	.27	.43	.31	.17	.26	.30	.23	.17	.24	.07	.16	.11	.15	.10	.08	.10	.13	.07	.05	.08	.02	.08
R. germanica	.23	.29	.32	.30	.30	.43	.24	.28	.22	.35	.29	.23	.33	.15	.07	.08	.14	.21	.09	.18	.12	.22	.06	.13	.22
R. hercynia	.22	.27	.21	.36	.21	.28	.30	.23	.28	.30	.31	.22	.07	.15	.14	.13	.13	.08	.08	.19	.10	.05	.07	.06	.20
R. hybrida	.31	.26	.13	.34	.31	.24	.27	.17	.29	.24	.29	.17	.06	.22	.17	.21	.12	.14	.16	.18	.08	.11	.08	.04	.15
R. insularis	.26	.22	.20	.33	.26	.15	.27	.34	.25	.29	.26	.15	.07	.26	.10	.27	.15	.21	.15	.19	.01	.01	.01	.02	.08
R. intermedia	.16	.11	.09	.18	.16	.09	.13	.02	.22	.20	.11	.05	.08	.30	.16	.24	.30	.14	.24	.22	.33	.16	.19	.17	.22
R. loyolaea	.29	.17	.17	.39	.29	.26	.30	.18	.35	.29	.28	.15	.19	.27	.16	.16	.16	.14	.21	.19	.15	.17	.13	.08	.21
R. nivata	.29	.36	.29	.34	.27	.25	.24	.34	.26	.34	.20	.19	.14	.35	.10	.29	.23	.19	.23	.17	.12	.12	.06	.05	.08
R. puthzi	.39	.25	.18	.30	.44	.31	.22	.23	.30	.25	.22	.25	.13	.24	.15	.22	.13	.15	.17	.20	.02	.05	.01	.06	.09
R. semicolorata ferruginea	.29	.19	.28	.25	.35	.52	.19	.21	.33	.33	.22	.28	.32	.15	.08	.02	.15	.15	.09	.15	.19	.21	.14	.16	.27
R. semicolorata iridina	.30	.22	.37	.25	.38	.53	.17	.24	.30	.28	.22	.28	.33	.16	.07	.02	.17	.15	.10	.15	.14	.21	.07	.16	.26
R. semicolorata picteti	.29	.20	.32	.25	.35	.54	.16	.26	.31	.32	.21	.30	.35	.16	.08	.02	.13	.16	.10	.15	.18	.23	.13	.13	.26
R. semicolorata semicolorata	.33	.21	.37	.27	.40	.53	.18	.26	.35	.35	.21	.30	.34	.17	.08	.02	.15	.15	.10	.15	.20	.25	.18	.16	.25
R. sibillina	.42	.34	.19	.30	.41	.34	.23	.23	.29	.17	.24	.29	.13	.20	.21	.19	.10	.12	.14	.16	.02	.06	0.0	.03	.16
Ec. alpinus	.86	.44	.63	.46	.78	.69	.41	.53	.69	.51	.40	.78	.17	.13	.19	.10	.03	.12	.13	.15	.07	.07	.13	.14	.30
Ec. angelieri	.47	.67	.56	.76	.45	.24	.84	.52	.52	.63	.74	.36	.20	.23	.20	.25	.23	.30	.36	.28	.14	.22	.19	.18	.17
Ec. aurantiacus	.49	.81	.61	.74	.48	.32	.84	.71	.55	.50	.71	.48	.28	.24	.19	.29	.25	.35	.38	.30	.14	.29	.23	.16	.18
Ec. bellieri	.55	.69	.64	.63	.55	.36	.71	.53	.60	.45	.59	.51	.19	.15	.23	.21	.17	.26	.28	.20	.14	.29	.26	.18	.22
Ec. carpathicus	.50	.51	.56	.70	.40	.48	.54	.56	.74	.65	.66	.44	.22	.17	.20	.21	.15	.17	.16	.28	.13	.34	.13	.15	.22

Table 7. Continued.

	hel	ins	kru	mac	par	pic	ruf	sta	sub	tor	ven	zel	gra	gri	hel	lat	ozr	pse	qua	vip	coe	dal	fla	sul	joe
Ec. dispar	.40	.44	.55	.44	.32	.29	.72	.57	.52	.61	.47	.38	.31	.09	.12	.09	.11	.27	.17	.12	.07	.30	.17	.18	.18
Ec. forcipula	.42	.61	.48	.83	.42	.22	.78	.53	.55	.58	.88	.32	.13	.22	.10	.20	.16	.27	.29	.22	.15	.25	.20	.24	.08
Ec. helveticus	–	.47	.56	.45	.93	.65	.40	.51	.71	.42	.41	.77	.08	.10	.11	.08	.04	.07	.10	.08	.07	.09	.13	.14	.29
Ec. insignis		–	.50	.65	.46	.39	.65	.57	.37	.40	.69	.48	.21	.29	.25	.40	.30	.31	.43	.30	.19	.21	.13	.16	.28
Ec. krueperi			–	.45	.47	.48	.45	.57	.50	.55	.44	.60	.27	.15	.27	.24	.18	.23	.19	.25	.14	.24	.20	.19	.24
Ec. macani				–	.42	.34	.72	.61	.61	.63	.82	.32	.14	.29	.12	.20	.17	.26	.29	.23	.15	.27	.13	.21	.11
Ec. parahelveticus					–	.67	.38	.41	.61	.34	.38	.70	.07	.09	.10	.07	.03	.07	.09	.06	.09	.09	.13	.15	.28
Ec. picteti						–	.26	.35	.48	.39	.30	.60	.21	.17	.16	.02	.10	.02	.10	.13	.08	.20	.08	.14	.51
Ec. ruffii							–	.62	.59	.55	.72	.39	.27	.16	.10	.20	.15	.40	.29	.22	.07	.23	.16	.10	.19
Ec. starmachi								–	.55	.49	.59	.52	.33	.09	.11	.20	.16	.33	.22	.21	.06	.20	.06	.14	.16
Ec. subalpinus									–	.59	.52	.54	.13	.10	.17	.13	.10	.13	.10	.15	.06	.28	.19	.12	.16
Ec. torrentis										–	.54	.32	.31	.24	.10	.11	.11	.26	.17	.18	.10	.25	.16	.18	.14
Ec. venosus											–	.35	.13	.23	.11	.20	.17	.27	.29	.23	.13	.28	.13	.22	.19
Ec. zelleri												–	.23	.03	.23	.14	.11	.15	.17	.20	.09	.09	.09	.12	.36
El. grandiae													–	.33	.40	.43	.41	.60	.39	.53	.20	.20	.15	.13	.27
El. gridellii														–	.49	.66	.64	.57	.87	.65	.38	.32	.32	.30	.25
El. hellenica															–	.75	.59	.48	.55	.72	.25	.33	.38	.19	.37
El. lateralis																–	.70	.69	.66	.77	.26	.26	.32	.19	.24
El. ozrensis																	–	.46	.71	.86	.38	.25	.38	.30	.32
El. pseudograndiae																		–	.57	.60	.18	.26	.26	.13	.17
El. quadrilineata																			–	.73	.38	.32	.32	.30	.32
El. vipavensis																				–	.31	.26	.39	.27	.33
H. coerulans																					–	.62	.61	.64	.17
H. dalecarlica																						–	.61	.55	.23
H. flava																							–	.54	.15
H. sulphurea																								–	.21
N. joernensis																									–

distinction of these two groups. We did not find any evidence for the subspecies rank of these two groups. It should be noted that, according to the morphological characteristics the six populations, can be assigned to *E. venosus*.

The highest identity coefficient was calculated between *E. forcipula* and *E. venosus* (Tables 2 and 7, specific electromorphs at the Apk- and Pgm-loci). *E. macani* which was a new record for Italy, was closely related to both species. Despite a comparison with paratypic material, it was difficult to assign these populations of *E. macani*. The abdominal patterns were much clearer on the male paratype (as determined by THOMAS), than on the individuals from Italy. The concave outer edge on the inner sclerite of the penis as described by THOMAS (THOMAS, 1970: 78, Fig. 5) is missing in the paratype, but is distinctly present in individuals from Italy.

Two populations occurring in South France (Asse and Lot), which are biochemically recognizable as separate taxa, could be assigned either to the species *E. angelieri* or to *E. ruffii*. Difficulties were encountered due to the poor description of *E. ruffii*. The drawings of the penis by GRANDI (1952) and FONTAINE (1964) are incorrect. Both taxa have a very similar penis form and structure; this similarity is also reflected by the high Ī-values in the matrix (Table 7) and in the dendrogram (Fig. 1). A redescription will bring clarity. An exact assignment of the two populations was made possible by using reference material from the collection of THOMAS.

Our larval material of *E. insignis* could be morphologically divided into two groups. During the larval stage both groups showed the typical sternite pattern for "*insignis*". However one of the groups could be easily identified by the characteristic white spots on the abdomen (occasionally also on head and thorax). The biochemical analysis revealed no significant differences between the two groups. This confirms the results of KIMMINS (1942) who also found these white-spotted nymphs in *E. dispar*, *E. forcipula* and *E. torrentis*.

E. dispar and *E. torrentis* are linked by the lowest identity coefficient among the *E. venosus* taxa. Many calculated Ī-values are lower than 0.50 (Table 7). None of these species show a particularly high affiliation with the other species, with the exception of *E. dispar* to *E. ruffii* (Ī-value 0.72).

Electrogena

The unsatisfactory assignment of the "*lateralis*"-group to either the genus *Ecdyonurus* or originally to the genus *Heptagenia* could be clarified by our biochemical investigations. The results from the analysis of eight studied "*lateralis*" species are sufficient evidence for the generic status of this group. This new genus *Electrogena* ZURWERRA & TOMKA, 1985 has already been reported in an earlier study together with a key for the determination of the three genera *Ecdyonurus*, *Electrogena* and *Heptagenia* (TOMKA & ZURWERRA, 1985). The sup-

porting biochemical evidence which allowed to establish the new genus is described below. The swarming behaviour is a further evidence for the generic rank. The representatives of *Electrogena* dance differently from the species of *Ecdyonurus* and *Heptagenia*, which show similar dancing movements (CHRISTIAN FISCHER, personal communication, 1986).

The genus *Electrogena* was separated from all other genera investigated by an Ī-value of 0.22 ± 6 (see dendrogram, Fig. 1 and correlation matrix, Table 7). The highest Ī-values were found for *Heptagenia* and *Nixe*. These exceeded only slightly the identity values to other genera (0.17—0.19). In addition, representatives of the genera *Iron* and *Cinygmula* from North America (see ZURWERRA & TOMKA, 1985: 101, Fig. 1) cluster also to a similar degree alike the genera analysed in this study. Most mayfly-investigators who have studied the "*lateralis*"-group, expressed the opinion that the "*lateralis*"-group belongs to the genus *Ecdyonurus*. However, our biochemical data contradict such an assignment. The Ī-value between *Electrogena* and *Heptagenia* was higher than the one between *Electrogena* and *Ecdyonurus*. For the genera *Electrogena* and *Heptagenia*, identical electromorphs were found at the Mdh-2-, Rdh- (except for *E. grandiae*), Ak- and Got-1 loci. In contrast, the genera *Ecdyonurus* and *Electrogena* shared common electromorphs only at the Apk- and Ipo-1-locus. A separation of the "*lateralis*"-group from the genus *Ecdyonurus* was evidenced by different allelic variants of enzymes like AK, α-GPDH, GOT-1, LAP, MDH-2, PGM and RDH (except *E. grandiae*). The genus *Electrogena* is characterized biochemically by a combination of electromorphs for the following enzymes:

MDH 2: electromorph 98	in common with *Heptagenia, Rhithrogena diaphana* and *R. intermedia*
α-GPDH: electromorphs 98,99	in common with *Rhithrogena* and *Nixe joernensis*
RDH: electromorph 99	with the exception of *E. grandiae* (electromorph 101); in common with *Heptagenia dalecarlica, H. flava* and *Epeorus alpicola* and *E. yougoslavicus*
IPO-2: electromorphs 101, 102	partially in common with *Ecdyonurus*
PGM: electromorphs 97, 98, 99	electromorph 99 in common with *Heptagenia coerulans*; electromorph 97 in common with *Epeorus alpicola*

The genus *Electrogena* can be separated by morphological criteria from *Ecdyonurus* and *Heptagenia* (ZURWERRA & TOMKA, 1985) and from other valid genera of the Heptageniidae in the Holarctic, Oriental and Ethiopian region as shown in the key to larvae and imagines (TOMKA & ZURWERRA, 1985).

Special attention must be paid to the similarities between the new genus and the genera *Afronurus* LESTAGE, 1924, and *Ecdyonuroides* DANG, 1967. Both

genera are clearly discernible from *Electrogena* by conspicuous morphological characteristics. These features, however, allow only to distinguish developmental stages. The larvae of *Ecdyonuroides* have long spiniform paranotal processes (TSHERNOVA, 1976: 48, Fig. 7) on their abdominal segments while *Afronurus* and *Electrogena* do not. The structure of the penis-lobes of imagines from the genus *Afronurus* is clearly different from that of the two other genera. The penis-lobes in *Afronurus* are medio-apically expanded and their ventral surface shows a pronounced longitudinal emargination (see SCHOONBEE, 1968). Since the species of the genera *Afronurus*, *Electrogena* and *Ecdyonuroides* have not always been accurately described, most features had to be tested for their validity as diagnostic characteristics. In their key to the genera of the Heptageniidae, TOMKA & ZURWERRA (1985) used the relative length of the first two tarsal articles and the contiguity of the eyes in male imagines to separate *Afronurus*, *Ecdyonuroides* and *Electrogena*. Following reexamination of more taxa, these features were found to be inadequate.

The eight *Eletrogena* taxa that were investigated biochemically are represented in Fig. 1. The assignment of the various populations to any of the known *Electrogena* species (until now described as members of *Ecdyonurus* or *Heptagenia*) was not problematic, with the exception of those from three localities. Upon reexamination and comparison of morphological features the populations found in the rivers Figarella (F, Corsica), Kucis (GR) and Vipava (YU) (see ZURWERRA & TOMKA, 1985) could not be assigned to any valid species. All three taxa will be described morphologically in a separate paper as the new species *E. hellenica*, *E. pseudograndiae* and *E. vipavensis* (ZURWERRA & TOMKA, 1986).

The highest $\bar{I}$-values within the genus *Electrogena* were obtained for the species pairs *E. gridellii* — *E. quadrilineata* and *E. ozrensis* — *E. vipavensis* (Fig. 1, Table 7). The first pair was distinguishable using the enzymes APK and HK-2, while the second pair is distinguished by different electrophoretic mobilities of the enzymes GOT-2, PGM and LAP. *E. vipavensis* from Yugoslavia could be directly compared on morphological grounds with *E. ozrensis* in both larval and imaginal stage since we have found for the first time the larvae of the latter species (details were published elsewhere).

The second new species *E. hellenica* is very closely related to the type species of the genus *Electrogena*, *E. lateralis*. The $\bar{I}$-value of 0.75 is based on allelic differences of the enzymes ALD, GOT-2, HK-1 and HK-2.

The relatively low similarity of *E. grandiae* — *E. pseudograndiae* to the rest of the *Electrogena*-taxa is documented in Fig. 1. *E. grandiae* showed the lowest genetic identity ($0.33 < \bar{I} > 0.60$, Table 7) to all species of *Electrogena* investigated. This is surprising considering the fact that it was only recently possible to distinguish this species morphologically from *E. lateralis* (BELFIORE, 1981). *E. pseudograndiae* has the highest identity to *E. lateralis* ($\bar{I} = 0.69$, Table 7), and can easily be separated from the other *Electrogena*-taxa at the Mdh-1-locus.

Heptagenia

We have initiated the biochemical investigation of some members of the genus *Heptagenia* to demonstrate the generic rank of *Electrogena*. In addition we have proved the validity of *H. dalecarlica*, which was questioned by some authors. Our biochemical results (see Fig. 1, Table 7) are in agreement with the morphological investigations of SAARISTO & SAVOLAINEN (1980) who succeeded in establishing the species rank of *H. dalecarlica*.

Nixe

The result of the separation according to biochemical criteria of two Norwegian populations of *"Heptagenia" joernensis* was surprising. The pairwise identity coefficients (Table 7) with the remaining *Heptagenia* species (highest Ī-value = 0.23) indicated a distant relationship, which excluded this species as a member of the genus *Heptagenia*. The comparison with the remaining analysed Heptageniidae taxa showed Ī-values which were lower than 0.37, with the exception of *Ecdyonurus picteti* (Ī-value = 0.51). The dendrogram clearly shows that *"joernensis"* clusters at a similar rank with *Electrogena* and *Heptagenia*. Therefore, it could not be assigned to any of the genera assayed in this study. The comparison of the main electromorphs between *"joernensis"* and the *Heptagenia* species showed that the typical *Heptagenia* electromorphs (see Table 2) are not present at the Apk, α-Gpdh, Mpi and Got-1 loci. Furthermore, other allelic variants specific for *"joernensis"* could be found. These are also found in other genera (e.g. AK).

Biochemical analysis also showed that the genera *Iron* and *Cinygmula* can be excluded for *"joernensis"*. These results have been published elsewhere (ZURWERRA & TOMKA, 1985; ZURWERRA et al., 1986).

The unambiguous assignment of *"joernensis"* to the nearctic genus *Nixe* FLOWERS, 1980 was possible by comparing all larval and imaginal morphological features with those of the thirty-one valid genera that have been described up to date. BRAASCH (personal communication, 1983) shares the same opinion concerning the generic status of *Nixe joernensis*.

Discussion

Morphological and biochemical features are the result of evolutionary processes like mutation, selection and genetic drift. Only the realization of a novel evolutionary trait, e.g., the acquisition of an apomorphic feature, results in the division of a common ancestor into genetically distinct populations, thus leading to new phylogenetic lines (Ax, 1984). To clarify systematic and phylogenetic questions, it is necessary to know whether two lines are reproductively isolated or not (see introduction). Because we did not carry out crossbreeding ex-

periments with Heptageniidae, reproductive isolation is not evidenced by our investigations. A direct proof cannot be gained by either morphological or biochemical data. However, analysis of the expression of genes coding for enzymes is in some cases a more valid approach to clarify phylogenetic relationships than morphological studies. We used the relative electrophoretic mobilities of enzymes as genetic markers. The identification of enzymatic activities produced by single genetic loci can be carried out with low enzyme quantities, present in crude lysates. Subsequent to gel electrophoresis, visualization of the position of a given enzymatic activity inside the gel was achieved by selective histochemical staining. It has been shown (NEI, 1971; SHAW, 1970) that on the average 30—40% amino acid substitutions are necessary to detect (at a given pH, ionic strength and buffer constituent) significant differences in the electrophoretic mobility of an enzyme. Two electromorphs with the same electrophoretic mobility will be scored as being identical even though the amino acid sequences may actually be different.

Various investigations have shown that populations, which share a common gene pool, display a similar composition of their alleles (electromorphs). Different alleles only accumulate if an interruption of gene flow between two populations occurs. The degree of genetic diversity between two taxa (in the present study expressed as genetic identity coefficient $\bar{I}$) depends mainly on the timespan following the separation from a common gene pool.

Some information is lost by reducing arrays of frequency data to a single $\bar{I}$-value. Nevertheless, the $\bar{I}$-value is a reliable measure for the biochemical diversity of two taxa. The graphical display of the clustering algorithm is a dendrogram.

The genetic identity value ($\bar{I}$) can be converted into a genetic distance (defined as $D = -\ln \bar{I}$, see NEI, 1972). The logarithmic scale is useful to display large genetic differences. Using the formula $T = 5 \times 10^6 D$ of NEI, it is even possible to estimate the time of divergence of two taxa. However, certain assumptions are necessary. This formula is based on the rate of electrophoretically detectable codon changes per locus per year on an average of 10^{-7} (NEI, 1972, p. 74). The fact that the observed exchange rate is different and that the evolution pressure varies in time was neglected. This estimation is especially meaningful for all cases where a direct comparison with geological data can be made. Using the above formula one can deduce that the genera *Ecdyonurus*, *Electrogena* and *Rhithrogena*, have become separated from one another at least 6.5×10^6 years ago. This division of the European genera of Heptageniidae probably took place prior to the separation of the island of Corsica from the mainland (Oligocene, about 35 million years ago). This is suggested by the presence of all European genera of Heptageniidae in Corsica, with the exception of *Arthroplea*, *Cinygma*, *Epeorus*, *Iron* and *Nixe*. The absence of *Epeorus* species on the island is astonishing, however, since representatives of this genus

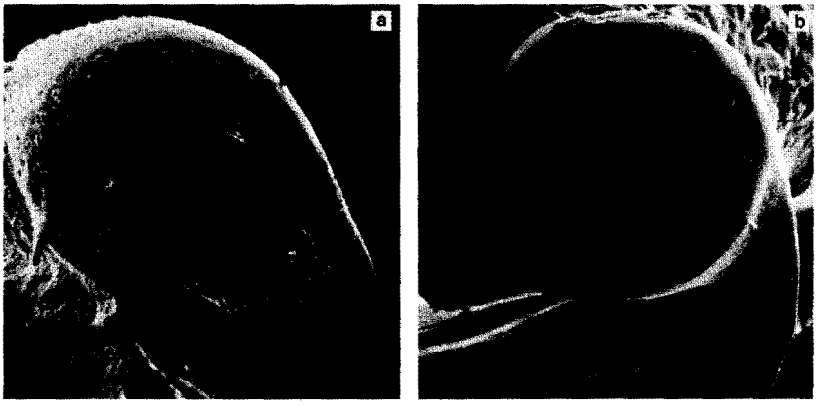

Fig. 3. SEM photographs of penis in apical view. (critical point drying method, gold coating, 12 kV) a) *Rhithrogena leavigata*-group (*R. semicolorata picteti* comb. n.), b) *R. lobata*-group (*R. hybrida*).

are widely distributed in the entire South European area. On the other hand the genetic divergence values between the endemic species of Corsica and those of the continent are not lower than the values for the continental species themselves. This observation argues for an immigration at a later period across some residual land bridges between Corsica and the mainland. It is generally accepted that the island of Corsica was covered with alpine layers from the Italian part of the continent. The divergence times T that can be calculated for the three endemic species *Ecdyonurus bellieri, Electrogena pseudograndiae* and *Rhithrogena insularis* and their continental counterparts, with respect to their corresponding groups or genera, support the notion of active (land bridges) or passive (wind, vectors) immigration. A shortest time of divergence of 580,000 years has been calculated for *Ecdyonurus bellieri* and *E. aurantiacus*. The highest value of 5.7×10^6 years was found for *Rhithrogena insularis* and *R. intermedia*. From the high identity coefficient and accordingly low T-value for *Ecdyonurus bellieri* and *E. aurantiacus* we must assume that *E. aurantiacus* populations are present on the island. This is indeed confirmed by the literature (Kimmins, 1930). *Ecdyonurus dispar, E. starmachi* and *E. torrentis* must have been separated earlier from the remaining taxa of the *E. venosus*-group. This conclusion is based on the higher genetic divergence as revealed by the dendrogram and the matrix. A similar conclusion can be drawn for the *Rhithrogena lobata*-group. An analysis of the identity coefficients indicates that *R. alpestris, R. intermedia, R. loyolaea* and *R. nivata* represent a more ancient monophyletic line than the other representatives of this group.

Electrogena pseudograndiae shows a similar relationship to *Electrogena* species with the exception of *E. hellenica* and *E. ozrensis* (Table 7). The fact that the Ī-values of the Balkan species are lower indicates that they represent another

phyletic line which may have originated in the Caucasus (see BRAASCH, 1983). However, it must be stressed that such a conclusion is only relevant in a statistical sense and strongly depends on the enzymes selected for analysis. The sixteen enzymes investigated evolve at different rates. To account for this, the degree of polymorphism was also considered for the listing of the enzymes in Table 2. Polymorphic enzymes, like PGM, GOT-1 and LAP, evolve more rapidly than others and are, therefore, more useful for the discrimination of species. However, conservative enzymes are a more reliable measure for establishing higher orders of phylogenetic relationships. It should be pointed out that the relationships determined by biochemical methods for the Heptageniidae species and groups are in good agreement with results from other methods. This indicates that our choice of the sixteen enzymes was an appropriate one. The reliability of the Ī-values for establishing generic ranks can be demonstrated by comparing the results from biochemical analysis and those from the analyses of morphological features (see introduction).

The dendrogram of all Heptageniidae taxa clearly shows two distinct lines: 1. *Epeorus, Rhithrogena, Ecdyonurus*, 2. *Electrogena, Heptagenia, Nixe*. Both, JACOB and JENSEN & EDMUNDS have always combined *Ecdyonurus* and *Heptagenia*. This classification was supported by a series of plesiomorphic larval features such as tracheal gills which, apparently, have been better conserved during evolution than enzymatic markers. At this point it should be mentioned that phylogenetic assignments with our biochemical method are only valid without restriction to Ī-value levels of 0.20 ± 5. Reliable relationships can only be determined by including all known worldwide distributed genera while the cluster analysis developed by NEI for closely related taxa only allows precise predictions within genera or groups. The value of the identity coefficient decreases with decreasing Ī-value. The significance of the identity coefficient can be raised by increasing the number of conservative loci studied. A statistical weighting of the enzymes, which should correlate with their evolutionary conservation could produce more significant results even at low Ī-values. However, this is problematic because the rate of mutation is known only for a few enzymes. In general, polymorphism increases with the number of individuals investigated. The low Ī-values associated with highly polymorphic enzymes like PGM or GOT-1 may erroneously simulate separation of two populations (see populations of *Ecdyonurus venosus* and *Rhithrogena semicolorata*-complex). However, investigations of AYALA & ANDERSON (1973) of the Mdh-2-locus show that the frequency at which an allele occurs in a population may be influenced by environmental factors and that different allelic variants of a locus may be an advantage for a species. This then means that all local populations are in their own equilibrium.

Despite of an extensive search for material, not more than ten individuals were on the average available for biochemical analysis for twelve out of a total

of fifty-five taxa. However, the results on the remaining forty-three taxa clearly demonstrate that investigation of larger number of individuals has little influence upon the significance of genetic identity values. Several investigators (GORMAN & RENZI, 1979; NEI, 1978) have shown that genetic similarities are far more affected by the number of sampled loci than by the number of sampled individuals.

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