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An electrophoretic and morphological study of three *Ecdyonurus* species (Ephemeroptera: Heptageniidae) occurring in the British Isles

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ABSTRACT. The objective was to investigate the validity of three closely-related British species: *Ecdyonurus dispar*, *E. venosus* and *E. torrentis*. The species were characterized by eleven enzyme-substrates and fifteen different enzyme-loci, comparisons being made not only between species but also between five populations of *E. dispar* (three from Britain, one from France, one from Switzerland), two populations of *E. venosus* and two populations of *E. torrentis* (one from Britain, one from Switzerland for both species).

Four monomorph enzyme-loci (aldolase, mannose phosphate isomerase, arginine phosphokinase, glutamate-oxaloacetate transaminase-2) exhibited interspecific differences in their mobilities and therefore validated the conclusion that *E. dispar*, *E. venosus* and *E. torrentis* are distinct species. There were no monomorph enzyme-loci that were different between populations of the same species. There were, however, some intraspecific differences revealed by the presence of polymorphic enzyme-loci: seven in *E. dispar* (retinol dehydrogenase, hexokinase-1 and 2, glutamate-oxaloacetate transaminase-1, malate dehydrogenase-1, phosphoglucomutase, indophenol oxidase-2), three in *E. venosus* (glutamate-oxaloacetate transaminase-1, malate dehydrogenase-1, phosphoglucomutase) and three in *E. torrentis* (hexokinase-1, glutamate-oxaloacetate transaminase-1, malate dehydrogenase-1).

The morphological characters of larvae and adults were examined and some were used in new keys to larvae and adults.

Introduction

The check list of the British Ephemeroptera lists four species in the genus *Ecdyonurus*: *E. dispar* (Curt.), *E. venosus* (Fab.), *E. torrentis* Kimm. and *E. insignis* (Etn.) (Elliott & Humpesch, 1983). Larvae and adults of *E. insignis* are easy to separate from those of the other three species (Macan, 1979; Elliott & Humpesch, 1983), but the morphological characters for separating the latter species are rather unreliable. This problem prompted Harker (1986) to analyse some of these morphological characters more critically and she suggested that *E. dispar*, *E. venosus* and *E. torrentis* could be one species with the variable morphological characters determined by changes in environmental condi-

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tions. In contrast, Humpesch (1980a) showed that the relationship between water temperature and duration of the embryonic development of all three species was described by a power function that varied significantly between the species. There were no intraspecific differences between two lake populations of *E. dispar*, but a marked difference between the two latter populations and a river population of *E. dispar* (see Fig. 3 in Humpesch, 1980a). It is possible that the river population was a different species that was not recognized because of taxonomic inadequacies (Elliott & Humpesch, 1983).

Apart from morphological characters, species and populations can be separated by studying their genetic expressions, using enzyme electrophoresis. This method has already provided some useful information for fifty-five species of Heptageniidae from continental Europe (Zurwerra & Tomka, 1985; Zurwerra *et al.*, 1986, 1987; Hefti *et al.*, 1986, 1987) and two species of Siphlonuridae (Söderström & Nilsson, 1986). The three *Ecdyonurus* species were therefore investigated in the same way to clarify their taxonomic status.

Material and Methods

Male last instar larvae were collected from the following locations: *E. dispar* from River Sarine (CH), River Loire (F), River Lune (GB), Ennerdale Water and Windermere (GB); *E. venosus* from River Gottéron (CH) and River Lune (GB) and *E. torrentis* from River La Broye (CH) and River Lune (GB). Adult stages were obtained by rearing the larvae in the laboratory. For morphological investigations, wings, legs and genitalia of the male imago were cut off and preserved in 80% alcohol together with the exuviae of the last larval instar and subimago. The morphological characters for the determination of larvae and male adults of *E. dispar*, *E. venosus* and *E. torrentis* given in the Appendix were drawn from alcohol (80%) preserved specimens collected in England in the summer of 1986: *E. dispar* from Windermere, *E. venosus* and *E. torrentis* from the River Lune. As the male genitalia were taken from specimens reared in the laboratory, their form was not influenced by copulatory movements (see Harker, 1986). The rest of the body of each imago was put into its own plastic tube, placed in liquid nitrogen and frozen at −70°C until biochemical analysis could be carried out. As the method for the biochemical analysis was described in detail by Zurwerra *et al.* (1986), only a brief account is given here.

The electromorph mobilities of fifteen enzyme-loci were investigated using starch gel electrophoresis. The gels (Conaught starch hydrolysed) contained the following buffer systems for the different enzyme-loci:

(i) Tris borate EDTA pH 9 (Ayala *et al.*, 1972) for the enzyme-loci α-glycerophosphate dehydrogenase (α-GPDH), mannose phosphate isomerase (MPI) and retinol dehydrogenase (RDH);

(ii) N-(3-amino-propyl)-morpholine citrate pH 7 (Clayton & Tretiak, 1972) for the enzyme-loci aldolase (ALD), glutamate-oxaloacetate transaminase (GOT-1; GOT-2), hexokinase (HK-1; HK-2), indophenol oxidase (IPO-1; IPO-2), malate dehydrogenase (MDH-1; MDH-2) and phosphoglucomutase (PGM) and the same buffer system with slight modifications (Zurwerra *et al.*, 1986) was used at pH 6 for the enzymes adenylate kinase (AK) and arginine phosphokinase (APK).

The individual specimens were kept separate during the whole procedure. Each individual was first homogenized in ten volumes of 0.1 M Tris HCl buffer (pH 8) and centrifuged for 10 min at 8000 ***g***. 20 μl of the protein supernatant were then placed in the slots of each gel (one specimen per slot) and vertical electrophoresis was applied at 12 V/cm at 4°C for 5–8 h depending on the buffers used. After the migration, specific staining systems were applied to indicate the position of each enzyme, based on the enzyme-substrate specific reactions: tetrazolium salt system (Harris & Hopkins, 1976) for all the investigated enzymes with the exception of GOT-1 and GOT-2, which were indicated with the fast-blue dye.

Analysis of the zymogram

The zymogram patterns were recorded photographically and the distance of the individual electromorphs from the slot was measured. The electrophoretic mobilities of the electromorphs were assessed in relation to individuals from a reference population (*Epeorus sylvicola* (Pict.): La Broye/Châtel-St-Denis, CH, 705 m) and were expressed as the

TABLE 1. Electromorph frequencies of fifteen different enzyme-loci (for abbreviations, see p. 162); showing the enzyme-loci (Enz.) used, the relative mobility index of the electromorphs (Elec.) with their frequencies (in %); (a) for five populations of *Ecdyonurus dispar*: Sarine, CH (Sar.); Loire, F (Loi.); River Lune, GB (R.L.); Windermere, GB (Win.) and Ennerdale Water, GB (En.), (b) for two populations of *E.venosus* (*E.ven.*): Gottéron, CH (Got.) and River Lune, GB (R.L.) and (c) for two populations of *E.torrentis* (*E.torr.*): La Broye, CH (Bro.) and River Lune, GB (R.L.). *n*=number of specimens analysed for each enzyme locus.

Enz.	Elec.		Populations of:								
			(a) *E.dispar*					(b) *E.ven.*		(c) *E.torr.*	
			Sar.	Loi.	R.L.	Win.	En.	Got.	R.L.	Bro.	R.L.
AK		*n*	*11*	*10*	*21*	*17*	*10*	*5*	*5*	*8*	*3*
	106		100	100	100	100	100	100	100	100	100
GPDH		*n*	*11*	*10*	*22*	*17*	*10*	*5*	*5*	*8*	*3*
	100		100	100	100	100	100	100	100	100	100
IPO-1		*n*	*10*	*9*	*17*	*12*	*10*	*5*	*5*	*8*	*3*
	99		100	100	100	100	100	100	100	100	100
MDH-2		*n*	*11*	*10*	*22*	*17*	*10*	*5*	*5*	*7*	*3*
	100		100	100	100	100	100	100	100	100	100
ALD		*n*	*11*	*10*	*22*	*17*	*10*	*5*	*5*	*8*	*3*
	96		0	0	0	0	0	100	100	100	100
	98		100	100	100	100	100	0	0	0	0
MPI		*n*	*11*	*10*	*21*	*17*	*10*	*5*	*5*	*8*	*3*
	101		0	0	0	0	0	100	100	100	100
	102		100	100	100	100	100	0	0	0	0
APK		*n*	*11*	*10*	*21*	*16*	*8*	*5*	*5*	*8*	*3*
	96		0	0	0	0	0	100	100	0	0
	98		0	0	0	0	0	0	0	100	100
	100		100	100	100	100	100	0	0	0	0
GOT-2		*n*	*11*	*10*	*21*	*17*	*10*	*4*	*5*	*8*	*3*
	97		0	0	0	0	0	100	100	0	0
	98		100	100	100	100	100	0	0	0	0
	99		0	0	0	0	0	0	0	100	100
RDH		*n*	*11*	*10*	*22*	*17*	*9*	*5*	*5*	*8*	*3*
	100		55	80	18	59	78	100	100	100	100
	101		45	20	82	41	22	0	0	0	0
HK-1		*n*	*13*	*11*	*26*	*20*	*11*	*5*	*5*	*9*	*3*
	101		23	9	42	50	45	0	0	22	0
	102		0	0	0	0	0	100	100	0	0
	102.5		77	91	58	50	55	0	0	78	100
GOT-1		*n*	*11*	*10*	*26*	*19*	*10*	*5*	*5*	*9*	*3*
	96		0	0	0	0	0	0	0	11	0
	101		0	0	0	0	0	20	0	0	0
	105		100	100	96	95	100	80	100	89	100
	108		0	0	4	5	0	0	0	0	0
MDH-1		*n*	*17*	*15*	*5*	*5*	*2*	*7*	*5*	*9*	*3*
	95		0	0	0	0	0	0	0	67	0
	97		0	0	0	0	0	0	0	33	67
	98		47	53	0	0	0	57	0	0	0
	100		53	47	100	100	100	43	100	0	33
PGM		*n*	*13*	*12*	*28*	*19*	*14*	*6*	*6*	*8*	*3*
	100		69	67	61	16	43	0	0	0	0
	101		31	33	39	84	57	0	17	100	100
	102		0	0	0	0	0	83	83	0	0
	103		0	0	0	0	0	17	0	0	0
HK-2		*n*	*12*	*10*	*19*	*15*	*10*	*5*	*5*	*7*	*3*
	105		0	0	0	0	20	0	0	0	0
	108		25	20	37	47	30	100	100	0	0
	110		75	80	63	53	50	0	0	100	100
IPO-2		*n*	*10*	*11*	*21*	*17*	*10*	*5*	*5*	*8*	*3*
	98		10	27	14	29	30	0	0	0	0
	99		0	0	5	6	40	0	0	0	0
	100		0	18	29	24	30	0	0	0	0
	102		90	55	52	41	0	100	100	100	100

relative mobility index (RMI; Zurwerra *et al.*, 1986) in contrast to the standardized method of measurement by Ayala *et al.* (1972). This index has the advantage of diminishing the effects of arbitrary fluctuations of variables such as temperature and gel heterogeneity during the electrophoretic run. The reproducibility of the method dictates the selection of the units of this

TABLE 2. Identity matrix of relative mobilities of fifteen enzyme-loci between five populations of *Ecdyonurus dispar*, two populations of *E. venosus* and two populations of *E. torrentis*; showing the location where the specimens were obtained and the coefficient of genetic identity ($\bar{I}$) between populations.

Species	Localities	Population number								
		1	2	3	4	5	6	7	8	9
E. dispar	(1) Sarine	–	0.99	0.94	0.92	0.90	0.52	0.53	0.61	0.58
	(2) Loire		–	0.92	0.91	0.92	0.51	0.53	0.62	0.59
	(3) River Lune			–	0.96	0.94	0.47	0.45	0.57	0.56
	(4) Windermere				–	0.97	0.51	0.48	0.61	0.60
	(5) Ennerdale W.					–	0.48	0.46	0.59	0.59
E. venosus	(6) River Lune						–	0.97	0.64	0.62
	(7) Gottéron							–	0.62	0.61
E. torrentis	(8) River Lune								–	0.97
	(9) La Broye									–

TABLE 3. Summary on morphological characters used to separate the three *Ecdyonurus* species in the larval and adult stages.

	E. dispar	*E. torrentis*	*E. venosus*
Larva			
Head capsule (Fig. 2a)	Rectangular	Rectangular (Kimmins, 1942)	All sides rounded (Kimmins, 1942)
Lateral margin of pronotum (Fig. 2b)	Strongly curved (proportion α/β >4.5)	Slightly curved (proportion α/β <4.0)	Slightly curved (proportion α/β <4.0)
Mouthparts: Mandible (Fig. 2d) Labium (Fig. 2e)	Setae of prostheca <10	Setae of prostheca <10	Setae of prostheca >12
Glossa (Fig. 2c)	Rounded	Elongated	Elongated
Male imago			
Genitalia ventral (Fig. 3a)			
Penis lobes	Triangular, rounded at the outer apex	Laterally elongated	Laterally elongated; pointed at the inner apex
Forceps base (Elliott & Humpesch, 1983)	Two pointed teeth curved inwards	Two blunt teeth not curved inwards	Dome-shaped with sometimes slight projections
Genitalia dorsal (Figs. 3b, 4)			
Penis lobes, apical margin of lateral sclerite	Curved distally	Not curved distally, parallel to the outer border of the penis lobus	Curved distally
Apical sclerite	Not prominent	Not prominent	Prominent
Subimago male and female			
Forewings (Kimmins, 1942; Elliott & Humpesch, 1983)	Uniformly greyish, cross-veins without strongly black borders	Mottled, with black borders to some cross-veins, showing four blackish bands across bands across the wing	Mottled, with black borders to all cross-veins

index (e.g. the resolution), which means that the RMI-value is given in integers, the unit of which expresses the minimum electrophoretic mobility between two electromorphs. In one case (HK-1) this resolution limit could be enhanced so that half of the digit unit could be used. If enzyme loci show the same RMI, they cannot be used to separate species. Monomorphic enzyme-loci allow statements about the genetic isolation between two populations when they exhibit different RMI-values for at least one enzyme-locus. Polymorphic loci, characterized by different RMI, represent only an intraspecific variation between two populations for a given enzyme-locus.

The frequency of the RMI was calculated for each enzyme of each population and was expressed as a percentage. The frequency values were used for the pairwise correlation of the populations, and this procedure produced an identity matrix defining Nei's genetic distance (Nei, 1972) between two populations. Finally the matrix was clustered according to the unweighted pair-group arithmetic average (UPGMA) method (Ferguson, 1980) to obtain a dendrogram ranging from zero (absolute genetic difference) to one (absolute genetic identity).

Results

Biochemistry

E.dispar, E.venosus and E.torrentis. Table 1 summarizes the distribution of the electromorphs for fifteen loci and their corresponding frequencies. The enzyme-loci AK, GPDH, IPO-1, MDH-2 were identical in their mobilities within the three species, but other monomorphic enzyme-loci ALD, MPI, APK and GOT-2 clearly indicated interspecific differences in their mobility. The enzyme-loci RDH, HK-1, GOT-1, MDH-1, PGM, HK-2 and IPO-2 were polymorphic.

Populations of E.dispar, E.venosus and E.torrentis. Table 1 clearly shows that the five populations of *E.dispar* (Table 1a), the two populations of *E.venosus* (Table 1b) and of *E.torrentis* (Table 1c) had no discriminative electromorph for all the monomorphic enzyme-loci. However, the polymorphic loci produced a definite degree of intraspecific variability in the different populations. A correlation matrix (Table 2), using the electromorph frequencies of all the different populations, showed high correlation values for intraspecific comparisons (the five populations of *E.dispar* and the two populations

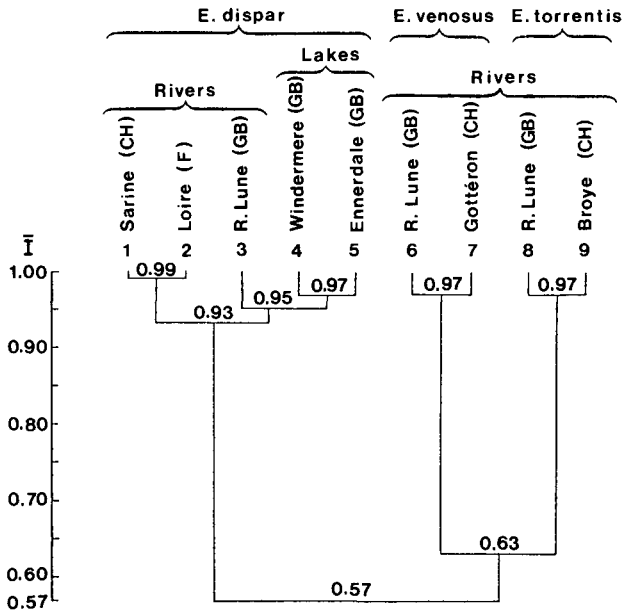

FIG. 1. Dendrogram of *Ecdyonurus dispar* (five populations), *E.venosus* (two populations) and *E.torrentis* (two populations) using the unweighted pair group arithmetic average (UPGMA) clustering method (Ferguson, 1980), where $\bar{I}$ expresses the mean genetic identity.

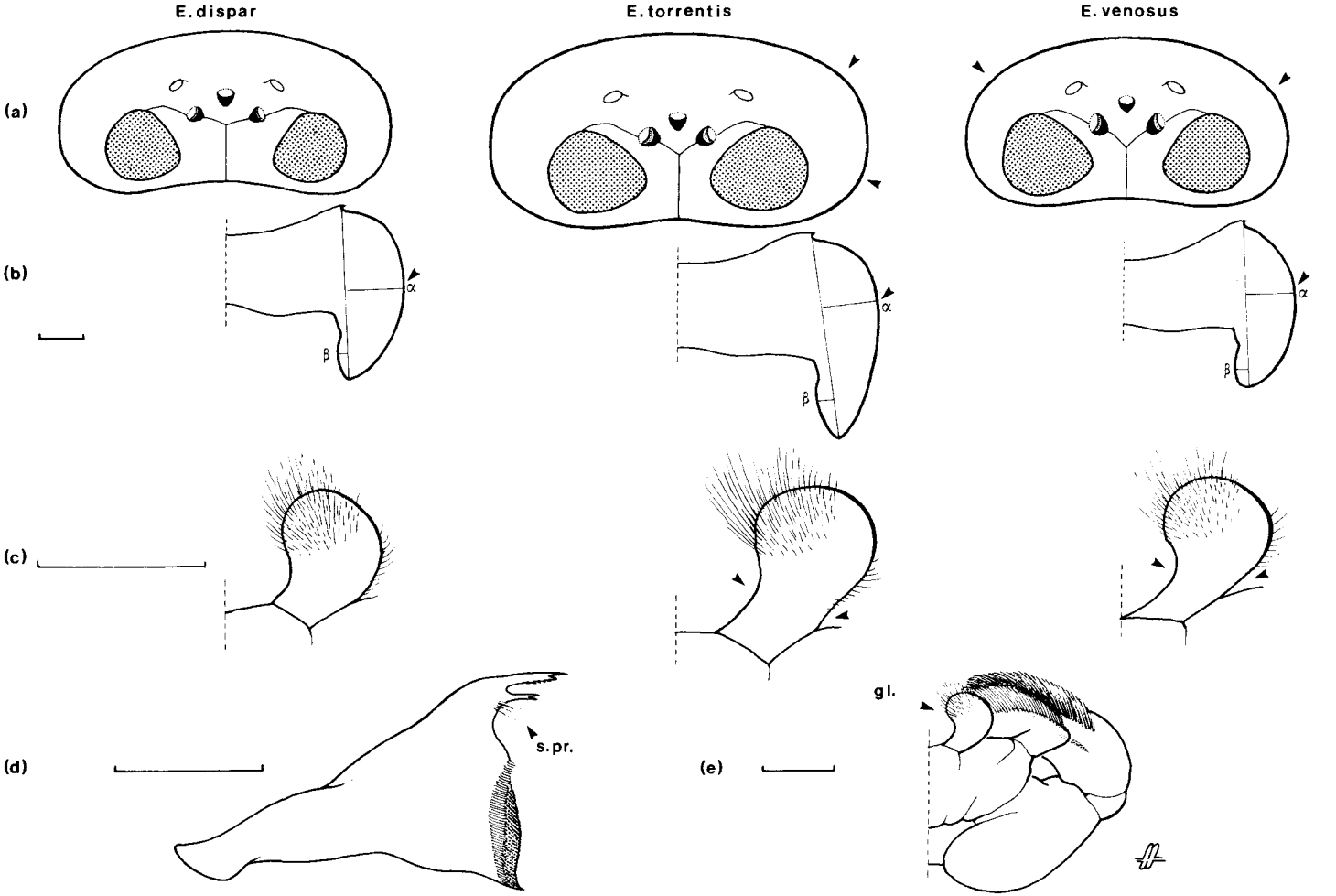

FIG. 2. Larvae of *Ecdyonurus* spp.: head capsule (a), pronotum (b) and glossa (c); *E. dispar*: Right mandible (ventral), showing the location of the prosthecal setae (d) and left side of labium (ventral, showing the location of the glossa (e); (s.pr.= setae of prostheca, gl.=glossa). Scale line 0.5 mm. (Original drawing by M. Mizzaro.)

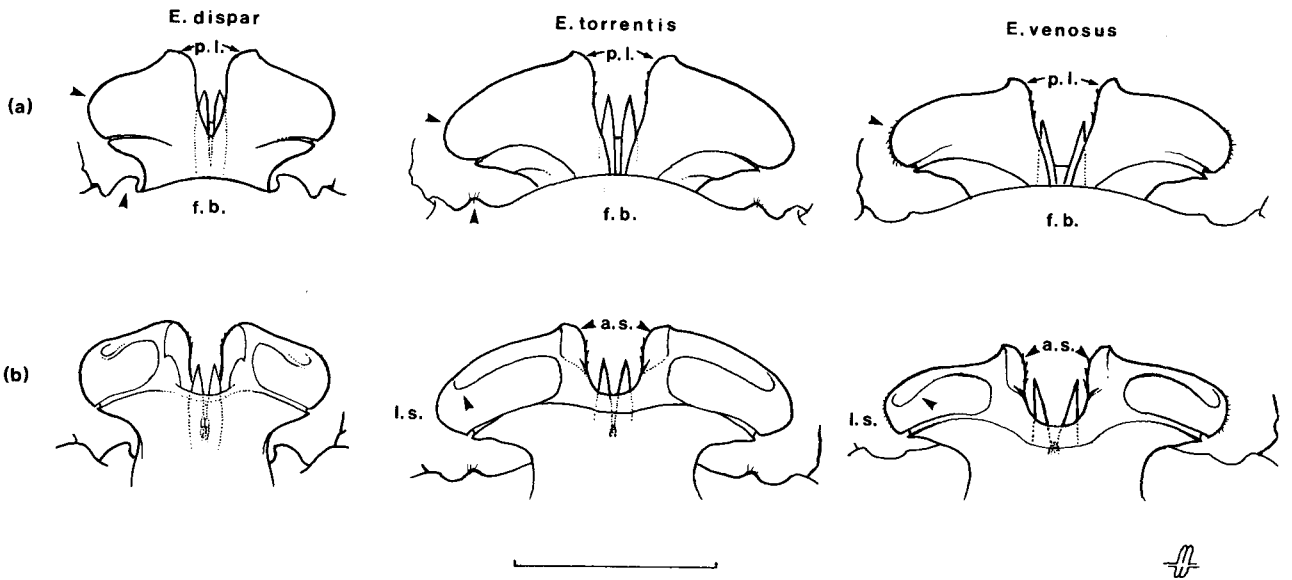

FIG. 3. Male genitalia of *Ecdyonurus* spp.: ventral (a), dorsal (b); f.b.=forceps base, p.l.=penis lobes, a.s.=apical sclerite, l.s.=lateral sclerite. Scale line 0.5 mm. (Original drawing by M. Mizzaro.)

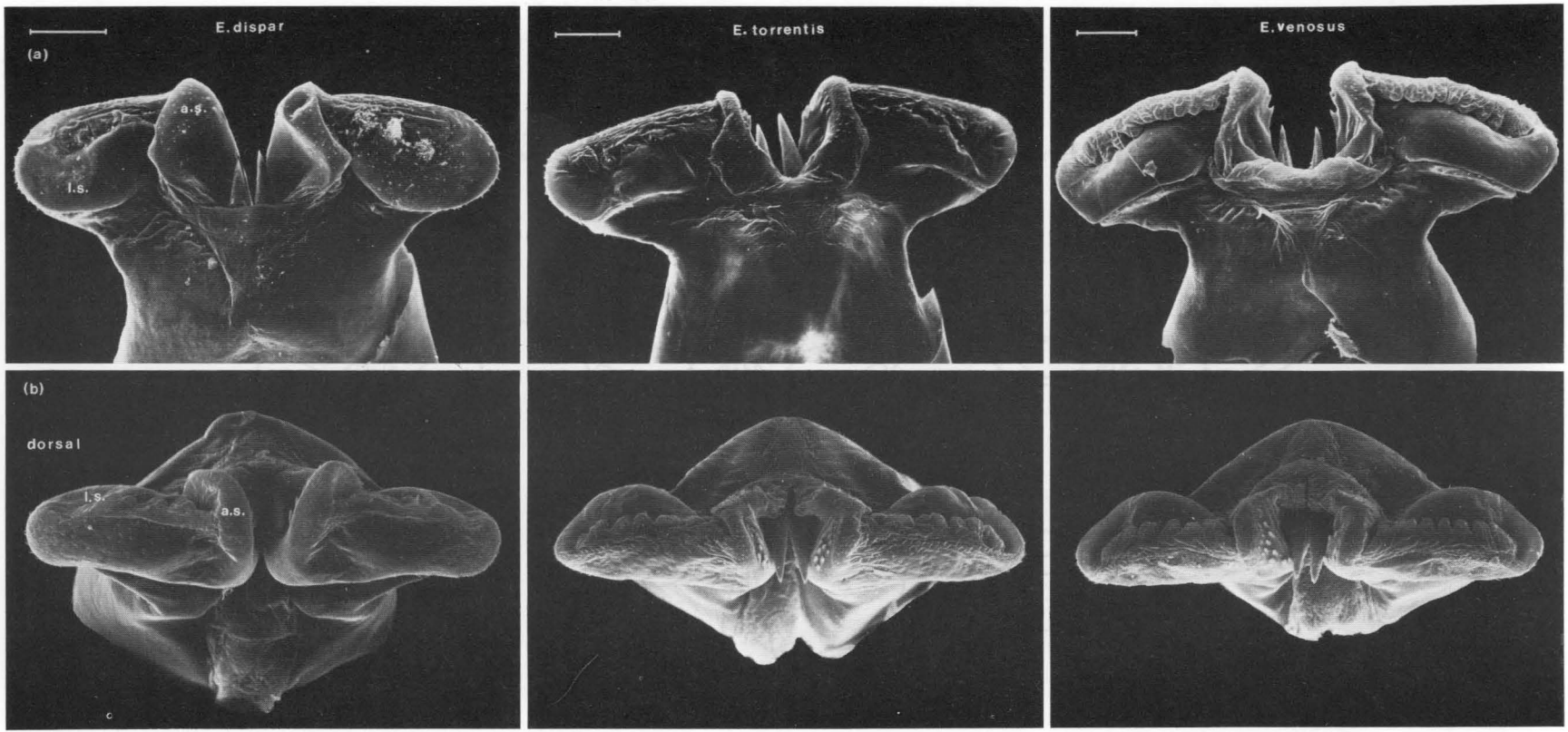

FIG. 4. Scanning electron microscope photographs of penis lobes of *Ecdyonurus* spp.: dorsal (a), apical (b); a.s.=apical sclerite, l.s.=lateral sclerite. Critical point dried, gold coated, 25 kV. Scale line 100 μm. (Photo by Müller and Landolt.)

of *E.venosus* and *E.torrentis*) but much lower values for interspecific comparisons.

Morphology

Larval and adult characters for separating the electrophoretically distinct species are summarized in Table 3. These characters were used to compile a new key to separate the three *Ecdyonurus* species (see Appendix).

Discussion

Recent investigations have demonstrated a statistical probability of about 0.4 (i.e. 40%) for the proportion of amino acid substitutions which can be detected by electrophoresis (Nei, 1971). If this criterion is fulfilled, there will be a significant difference in the RMI-value for different electromorphs of the same enzyme-locus. The different RMI-values of an enzyme (genlocus) within one population represent polymorphism. As more enzyme-loci are analysed electrophoretically, the higher is the certainty of the results. The absence of a common electromorph of an enzyme-locus characterizes genetic isolation in two populations and indicates the presence of two species. As differences were present in more than one enzyme for the species pairs in the present investigation (five between *E.dispar* and *E.torrentis*, six between *E.dispar* and *E.venosus* and six between *E.venosus* and *E.torrentis*), it is obvious that these taxa are distinct species.

On the basis of a statistical investigation of some larval and adult morphological characters, Harker (1986) was doubtful about the validity of the three *Ecdyonurus* species: *E.dispar*, *E.venosus* and *E.torrentis*. She suggested that the morphological differences were phenotypic rather than genotypic. However, the present investigation clearly shows that the three species are genetically distinct with low $\bar{I}$-values (Table 2). The morphological characters of the *Ecdyonurus* species examined by Harker (1986) are therefore not relevant for separating these three species. In contrast, the characteristics in Table 3 were found to be valid for a large number of specimens from a wide range of zoogeographical areas (continental Europe and Great Britain).

Both the genetical and morphological differences between species in the present study correlate well with ecological differences such as the duration of embryonic development (Humpesch, 1980a).

Table 1 clearly shows that the five populations of *E.dispar* and the two populations of *E.venosus* and *E.torrentis* have common electromorphs and therefore share the same genetic pool. The differences in their $\bar{I}$-values, appearing in the dendrogram (Fig. 1), derive only from different electromorph frequencies in polymorphic enzyme-loci.

According to the $\bar{I}$-values of *E.dispar*, the continental populations are separated from the British ones, and within the British populations the lake populations appear to be genetically closer to each other than to the river population (Fig. 1). These differences between the lake populations and the river population are also reflected in their different rates of embryonic development (Humpesch, 1980a). Both results may reflect the process of genetic isolation between populations living in different environments. The correct genetic interpretation of the polymorphic enzyme-loci could not be supported by cross-breeding experiments. Such experiments may be carried out in the future, because artificial insemination is possible in Ephemeroptera (Humpesch, 1980a; Humpesch & Elliott, 1980). There is the additional problem that some Ephemeroptera species are also parthenogenetic (Humpesch, 1980b).

This study therefore shows the importance of combining biochemical, morphological and ecological information to solve difficult taxonomic problems. A large amount of material, including specimens taken during the whole flight period as well as from consecutive seasons, is needed to improve the results.

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Appendix

Key to the larvae and adults of *Ecdyonurus dispar, E. torrentis* and *E. venosus*.

Mature larva (exuvia)

1 Lateral margins of the pronotum are strongly curved (proportion $\alpha/\beta > 4.5$) (Fig. 2b); glossa of the labium is short and round (Fig. 2c) ..... *dispar*

– Lateral margins of the pronotum are only slightly curved (proportion $\alpha/\beta < 4.0$) (Fig. 2b); glossa is elongated (Fig. 2c) 2

2 Head capsule is rectangular (Fig. 1a); less than 10 setae in prostheca (Fig. 1d) ..... *torrentis*

– Head capsule is rounded (Fig. 1a); more than 10 setae in prostheca..... *venosus*

Male imago

1 Penis lobes in ventral view are triangular and rounded at the outer apex and the forceps base has two pointed teeth curved inwards (Fig. 3a) *dispar*

– Penis lobes in ventral view are laterally elongated and the forceps base has no pointed teeth curved inwards (Fig. 3a) 2

2 Penis lobes in dorsal view with the apical margin of the lateral sclerite not curved distally, parallel to the outer border of the penis lobes (Figs. 3b, 4); forceps base with two blunt teeth not curved inwards (Fig. 3a) ..... *torrentis*

– Penis lobes in dorsal view with the apical margin of the lateral sclerite curved distally (Figs. 3b, 4); forceps base dome-shaped with sometimes slight projections (Fig. 3a) ..... *venosus*

[The subimaginal characters for both sexes given by Kimmins (1942) and Elliott & Humpesch (1983) have again been verified here for a large number of reared specimens from Austria, England and Switzerland.]