Ital. J. Zool., *63:* 23-30 (1996)

Estimating genetic variation from larvae and adults of mayflies: an electrophoretic analysis of three species of Heptageniidae (Ephemeroptera)

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ABSTRACT

Genetic variation in larvae and imagines of three species of Heptageniidae from central Italy, *Ecdyonurus* sp. *(venosus* group), *Electrogena grandiae* (Belfiore, 1981), and *Electrogena lateralis* (Curtis, 1834) was studied by starch-gel electrophoresis. Thirteen enzyme systems were analysed for a total of 20 loci (Aat, Ada, Adk-1, Adk-2, Ck-1, Ck-2, Est-1, Est-2, a-Gpdh, Gpi, La-1, La-2, Mdh-1, Mdh-2, Me-1, Me-2, Mpi, Pap, Pgm-1, Pgm-2), 15 of which were expressed in adults and 16 in the larvae. Only 11 loci (Aat, Ada, Est-2, Gpi, La-2, Mdh-1, Me-2, Mpi, Pap, Pgm-1, Pgm-2) were shared by larvae and adults. Observed heterozygosities were always lower than expected values; higher values were observed in larvae than in adults. Fixation indices and genetic distances were higher in the adults; however, the two genera were better discriminated using the larval samples. In conclusion, comparisons using larvae and adults simultaneously should be avoided, and absolute values of genetic identities and genetic distances to assess systematic levels have to be considered with caution, since they can vary from one developmental stage to another.

KEY WORDS: Genetic variation - Electrophoresis - Ephemeroptera - *Ecdyonurus - Electrogena.*

ACKNOWLEDGEMENTS

Many thanks are due to Mrs Emilia Giuliano (Foggia, Italy) for the revision of the English text and to two anonymous reviewers for the useful comments. This research was supported by a grant from the Italian Ministero della Università e della Ricerca Scientifica e Tecnologica, MURST.

{Received 31 May 1995 - Accepted 15 September 1995)

INTRODUCTION

Ephemeroptera usually have a complex life cycle *(sensu* Wilbur, 1980), with a long lasting aquatic larva and a short-lived adult stage with reproductive and dispersal functions. Traditionally, ephemeropteran systematics has been based on adult morphological features, and only recently has the importance of larval morphology been stressed (e.g., Belfiore & D'Antonio, 1991).

Studies of molecular evolutionary genetics in insects are numerous, and have been fundamental to the development of this discipline. From this point of view, however, several groups, like the Ephemeroptera, are still little studied. Until now, studies about mayflies have assessed genetic variation using electrophoretic techniques; most of these studies deal with systematics, i.e., tend to find diagnostic loci between taxa and to reconstruct their phylogenetic relationships (e.g., Zurwerra *et al.,* 1984, 1986, 1987; Søderstrøm & Nilsson, 1986; Funk *et al.,* 1988; Hefti *et al.,* 1988, 1989; Savolainen *et al.,* 1991; Belfiore *et al.,* 1992; Studemann *et al.,* 1994). Ferver studies deal with population genetic structure (Sweeney *et al.,* 1986, 1987; Sweeney & Funk, 1991).

The aforesaid studies are based mostly on adults (i.e., imagines), that are found only during restricted periods of the year. The collection of imagines may be difficult also because of the localization of their swarms and their flight height. Thus, just like in morphological studies, it would be very advantageous to employ the larvae for molecular studies: larvae indeed are easier to find and collect than adults; however, they undergo a number of moults, and morphological differences among instars are not easily detectable. It should be noted, furthermore, that a number of genes are expressed during different periods of embryonic and larval development (e.g., Bauer & Levenbook, 1969; Pantelouris & Downer, 1969; Counce, 1973; Bewley *et al.,* 1974; Wagner & Selander, 1974; Gasperi *et al.,* 1975; Kafatos *et al.,* 1977; Levenbook, 1986). With respect to the Ephemeroptera, Basha & Pescador (1984) and Matha & Šula (1984) reported differences in electrophoretic mobility of general proteins in the hemolymph of conspecific larval and adults stages of *Dolania americana* and *Ephemera danica,* respectively. Søderstrøm & Nilsson (1986) detected similar differences in leucine aminopeptidase enzymes between nymphs and imagines of two siphlonurid species, and found that the electromorphs of the two species differ only during the nymphal stage, whereas those of superoxyde dismutase enzyme never differ. Kownacki & Starmach (1984) did not find any variation between stages in the electrophoretic mobility of non-specific esterases in a number of Polish species and they suggested the use of these esterases to discriminate among species, particularly at early developmental stages.

The results of the above cited authors indicate that differences in protein electrophoretic mobility between

stages do exist, even if they seem to change from one locus to another. Subsequently, at present we ignore the effects they could have in a study of molecular population genetics or molecular systematics if different stages were employed together, or if similar results were obtained by comparing individuals at one stage or another. Thus, we used starch-gel electrophoresis to analyse a number of presumptive isozymic loci, separately in adults and larvae of three species of mayflies (Heptageniidae) from central Italy. Several genetic parameters were then inferred, and compared between stages and species in order to assess how different stages can be employed in electrophoretic studies.

MATERIALS AND METHODS

The following material was analysed:

Ecdyonurus sp. *(venosus* group): 15 male imagines and 20 larvae, Licenza (Roma), Licenza River, 380 m a.s.l., 18.7.1992 (see Belfiore & D'Antonio, 1991, for a discussion on the taxonomic status of species belonging to this group);

Electrogena grandiae (Belfiore, 1981): 27 male imagines and 27 larvae, Canale Monterano (Roma), Rafanello Stream, 250 m a.s.l., between 15.7.1992 and 4.8.1992;

Electrogena lateralis (Curtis, 1834); 32 male imagines and 26 larvae, Licenza (Roma), Licenza River, 380 m a.s.l., 18.7.1992.

To obtain samples approximately at the same developmental stage, larvae with about the same degree of wing pad development were selected. Genetic variation was estimated by horizontal starch-gel electrophoresis according to Murphy *et al.* (1990). Specimens were frozen in the field in liquid nitrogen and then homogenized in dH_20 (1:1 v/w) and stored in an ultra-cold freezer at -80° C until analysed. The liquid fraction of each homogenate was then loaded into a 10% starch gel (Sigma Potato Starch S4501) using small filter paper wicks. Electrophoresis was performed at +5° C. Isozyme systems were stained according to the histochemical techniques of Murphy *et al.* (1990). The assayed enzyme systems with their IUBNC nomenclature (1984), the employed buffers and migrating conditions are listed (Table I). If a given technique resulted in the appearance of more than one system of bands, these were Interpreted as products of presumptive isozymic loci and were labelled with progressive numbers starting with the fastest migrating. Within a given system, different bands were considered as allozymes and coded with letters, «a» being the fastest migrating. Preliminary investigations in our laboratory on a number of species did not detect any sex-linked difference in the examined loci.

Larvae and adults from the same population were considered as separate samples. The following measures of genetic variation for each sample were computed (Nei, 1987): mean sample size per locus, mean number of alleles per locus, percentage of polymorphic loci (a locus was considered polymorphic if more than one allele was scored), and mean heterozygosity observed (H_0). Genotype and allele frequencies were computed for each population; from the latter, the mean heterozygosities expected under Hardy-Weinberg (H-W) equilibrium (H_e) were estimated with Nei's correction for small sample sizes (Nei, 1978).

The hypothesis of H-W equilibrium was estimated for each polymorphic locus in each sample by Pearson χ^2 goodness-of-fit tests between the observed genotype frequencies and those expected under H-W equilibrium, computed using the formula of Levene (1949) for small samples. When more than two alleles were scored, the χ^2 tests were repeated by pooling the genotypes into three classes, i.e., homozygotes for the most common allele, heterozygotes for the most common allele and one of the others, and all other genotypes. The H-W hypothesis was also tested by calculating exact significance probabilities from 2 x 3 contingency tables for each locus, using the pooling procedure described above (e.g., Elston & Forthofer, 1977). Heterozygote excesses were also estimated for each locus by computing Wright's fixation indices (e.g., Nei, 1987).

Pearson χ^2 tests on contingency tables of both genotype and allele frequencies (Workman & Niswander, 1970) were performed between the two *Electrogena* species both for adult and larval stages, to assess significant heterogeneity. The same tests were repeated for each species between larvae and adults for the loci exhibited by both stages.

The critical probability value for each of the previous tests to reject the null hypothesis was set at $P < 0.05$.

The degree of genetic divergence between OTUs both for larval and adult stages was assessed by computing fixation indices (F_{ST}; Wright, 1978). F_{STS} were estimated both by G_{STS} (Nei, 1973) and ϑs (Weir & Cockerham, 1984), following the formulations of Slatkin (1993). Unbiased genetic distances, Ds, according to Nei (1978) were computed between OTUs.

TABLE I - *Electrophoretic conditions for the 13 enzyme systems evidenced in the three species of Heptageniidae.*

Enzyme system	Abbreviation	Number of loci	IUBNC code	Buffer system*	Migration conditions
Aspartate aminotransferase	Aat	1	2.6.1.1	A	637Vh/cm
Adenosine deaminase	Ada	1	3.5.4.4	B	62Vh/cm
Adenylate kinase	Adk	2	2.7.4.3	B	83Vh/cm
Creatine kinase	Ck	2	2.7.3.2	B	92Vh/cm
Carboxylesterase	Est	2	3.1.1.-.	A	213Vh/cm
Glycerol-3-phosphate dehydrogenase	a-Gpdh	1	1.1.1.8	B	92Vh/cm
Glucose-6-phosphate isomerase	Gpi	1	5.3.1.9	B	137Vh/cm
L-leucyl-L-alanyl peptidase	La	2	3.4.11.1	A	225Vh/em
Malate dehydrogenase NAD dep.	Mdh	2	1.1.1.37	B	90Vh/cm
Malate dehydrogenase NADP dep.	Me	2	1.1.1.40	B	137Vh/cm
Mannose-6-phosphate isomerase	Mpi	1	5.3.1.8	B	64Vh/cm
L-phenylalanyl-L-proline dipeptidase	Pap	1	3.4.13.9	A	256Vh/cm
Phosphoglucomutase	Pgm	2	2.7.5.1	C	206Vh/cm

*A, Lithium-borate, pH 8.1/ Tris-citrate, pH 8.2; B, Tris-citrate II, pH 8.0; C, Tris-maleate-EDTA, pH 7.4 (Murphy *et al.,* 1991).

TABLE II - *Genotype frequencies for the assayed presumptive loci in adults and larvae of the three species of Heptageniidae. Loci marked by an asterisk (*) were not evidenced in adults or larvae.*

Locus		Species					
		Ecdyonurus sp		*E. grandiae*		*E. Iateralis*	
		Adults	Larvae	Adults	Larvae	Adults	Larvae
Aat	(n)	7 cc	7 cc	9 bb	9 bb	9 aa	9 aa
Ada	(n)	13 aa(0.077) bb(0.154) cc(0.769)	18 ab(0.222) ac(0.278) be(0.278) cd(0.222)	14 aa(0.143) ab (0.071) bb(0.786)	15 ac(0.133) bb (0.133) be (0.733)	28 aa	24 ab (0.292) ac (0.667) cc (0.042)
Adk-1*	(n)	13 aa(0.692) ab(0.231) bb (0.077)	18	14 aa (0.714) ab(0.214) bb(0.071)	24	23 aa (0.565) ab(0.130) bb (0.304)	24
Adk-2'	(n)	13	5 aa (0.800) bb (0.200)	24	7 aa (0.429) bb(0.571)	30	5 aa (0.400) bb (0.600)
Ck-1*	(n)	12 aa (0.500) ab (0.250) bb (0.250)	18	15 aa (0.533) ab (0.200) bb (0 267)	24	27 aa (0.556) ab (0.259) bb(0.185)	24
Ck-2*	(n)	13	4 aa (0.500) bb (0.500)	23	4 bb	29	4 bb
Est-1*	(n)	10	17 aa(0.471) ab (0.293) ac(0.118) bb (0.059) be (0.059)	24	21 aa(0.191) bb(0.571) cc(0.238)	24	24 ab (0.542) ac (0.042) bb (0.250) cc(0.167)
Est-2	(n)	5 aa (0.200) bb (0.200) cc (0.200) fg (0.200) gg(0.200)	15 aa(0.133) ab (0.067) be (0.267) bd(0.133) be (0.200) cc (0.067) cd(0.133)	13 bb (0.077) be (0.308) cc(0.308) dd(0.308)	19 ac(0.210) bb(0.158) be (0.210) bd (0.053) dd(0.316) de(0.053)	12 aa(0.500) bb (0.500)	17 aa (0.235) ac (0.235) ae (0.059) bb (0.059) cc(0.176) cd(0.059) cc(0.176)
a-Gpdh*	(n)	4 aa	8	9 bb (0.222) cc (0.778)	9	10 aa (0.200) bb (0.700) be (0.100)	9
Gpi	(n)	15 aa(0.200) bb (0.667) bd (0.133)	20 aa (0.050) ab (0.800) ac (0.050) bb (0.050) be (0.050)	27 cc(0.407) cd(0.111) dd(0.370) ff(0.074) fg(0.037)	27 cc(0.333) cd(0.148) dd (0.370) dc (0.037) ff(0.111)	32 bb(0.031) cc(0.375) cd (0.094) dd (0.406) cf(0.031) ff(0.063)	26 cc (0.385) cd (0.038) dd (0.500) df(0.038) ff(0.038)
La-1*	(n)	5	6 aa	7	6 aa (0.667) bb(0.333)	6	6 aa (0.500) bb(0.500)
La-2*	(n)	3 aa(0.333) bb(0.333) ad (0.333)	4 aa(0.250) cc (0.250) dd (0.500)	3 bb	aa (0.200) bb (0.800)	4	4 bb (0.250) cc (0.750)
Mdh-1	(n)	13 ab (0.846) bb (0.077) cd (0.077)	13 ab (0.077) bb (0.385) bd (0.077) cc(0.154) cd (0.077) dd (0.231)	7 bc	9 ab(0.222) bb(0.111) bc (0.444) cc (0.222)	14 ab(0.071) be (0.286) cd (0.643)	13 bb (0.077) be (0.308) cc (0.077) cd(0.231) cc (0.077) dd(0.231)
Mdh-2*	(n)	12 aa(0.333) bb(0.417) bc (0.250)	18	10 aa	24	23 aa	22
Me-1*	(n)	12	17 aa	12	aa	25	22 aa (0.909) bb(0.091)
Me-2	(n)	12 aa(0.750) bb(0.250)	17 bb (0.647) cc(0.353)	12 bb(0.917) dd(0.083)	11 bb(0.455) cc(0.545)	25 aa (0.040) bb (0.960)	22 bb(0.273) cc (0.636) dd (0.091)
Mpi	(n)	7 aa(0.286) bb(0.571) cc (0.143)	8 bb (0.625) cc (0.375)	6 aa	7 aa	9 bb (0.444) cc(0.556)	10 aa(0.100) bb (0.400) cc (0.500)
Pap	(n)	13 bb	18 aa(0.278) bb(0.667) cc(0.056)	21 aa(0.381) bb(0.619)	20 aa	27 bb (0.963) cc (0.037)	24 aa(0.917) bb(0.083)
Pgm-1	(n)	7 aa(0.571) ab (0.286) bb (0.143)	10 aa (0.400) ab (0.400) bb (0.200)	10 be (0.400) cd (0.300) de(0.300)	10 bc (0.200) cc (0.200) cd (0.400) de (0.200)	10 bc (0.300) cc(0.100) cd (0.300) dd (0.200) ee(0.100)	11 bc (0.182) cc (0.273) cd (0.364) de(0.182)
Pgm-2	(n)	3 bb	3 aa	4 cc	3 bb	5 bb	4 aa (0.750) bb (0.250)

Ninety-nine-percent confidence limits for H_os, H_es, G_{ST}s, ϑs, and Ds were obtained by the bootstrap numerical resampling procedure (Weir, 1990): 1000 repeated samples for each population were constructed by random sampling with repetition over loci. The confidence intervals so computed were used for comparisons between larvae and adults of the same species.

Computations were made using BIOSYS-1 (version 1.7: Swofford, 1989) and a Microsoft QBASIC programme, BOOTSTR, written by one of us (G.S.).

RESULTS

The electrophoretic analysis detected 13 enzyme systems, for a total of 20 presumptive loci in each species, whose genotypic frequencies are given (Table II). Allele frequencies are not reported for the sake of brevity, since the reader can easily obtain them from the genotype frequencies. Another locus of Aat with anodal migration was not considered since it was poorly resolved. All loci showed variation among samples. Only 11 loci were detected in both adults and larvae: Aat, Ada, Est-2, Gpi, La-2, Mdh-1, Me-2, Mpi, Pap, Pgm-1, Pgm-2; therefore, comparisons between developmental stages in the same species were based only on these loci. Four loci were found exclusively in adults (Adk-1, Ck-1, a-Gpdh, Mdh-2) and five were restricted to larvae (Adk-2, Ck-2, Est-1, La-1, Me-1), for a total of 15 loci detected in the adult array and 16 in the larval one. In the adults of *E. Iateralis*, the La-2 locus was not evidenced, and it was not considered in the comparison of this population with their larvae or with the other species. Descriptive statistics of genetic variation in adults and larvae are given (Table III). Many parameters have different values between stages: the mean number of alleles per locus is the same for both stages of *Ecdyonurus* sp., and slightly higher in the larvae of the two *Electrogena* species; the percentage of polymorphic loci is in general rather high, and higher in the larvae (when compared with adults) in *Ecdyonurus* sp. and *E. Iateralis*, whereas the opposite was found for *E. grandiae*. Observed heterozygosities were always lower than those expected under H-W equilibrium; both values are higher in the larvae, except in *E. grandiae*. Mean heterozygosities and their confidence limits computed by the bootstrap procedure are given (Table IV): each value was significantly different from the others, since their confidence intervals did not overlap. Table V show the results of the tests for H-W equilibrium. In the adults, in most cases deviations from H-W equilibrium were caused by heterozygote deficiencies; only the Mdh-1 and Pgm-1 (for only one test in *E. grandiae)* loci showed significant heterozygote excesses. A similar situation was found for the larvae: a significant excess of heterozygotes was observed at Ada for all species, and at Gpi and Est-2 for *Ecdyonurus* sp. only. It is worth noting that the Mdh-1 locus in the larvae showed heterozygote deficiencies for *Ecdyonurus* sp., while it resulted in H-W equilibrium for the other species, whereas in the adults an excess of heterozygotes was always found. In contrast, the Ada locus showed a significant excess of

TABLE III - *Descriptive statistics of genetic variation in the three species of Heptageniidae. Abbreviations as follows: S, Mean sample size per locus; A, mean allele number per locus; P, % of loci polymorphic; H_o, mean heterozygosity observed; H_e, mean heterozygosity expected under Hardy-Weinberg equilibrium, corrected for sample size. Values in brackets represent standard deviations.*

Species	Stage	S	A	P	H_o	H_e
Ecdyonurus sp.	Adults	9.3(11)	2.4(0.3)	73.3	0.174(0.063)	0.392(0.073)
	Larvae	11.4(15)	2.4(0.3)	75.0	0.238(0.091)	0.429(0.070)
E.grandiae	Adults	11.6(16)	2.0(0.3)	66.7	0.196(0.088)	0.311 (0.070)
	Larvae	11.5(17)	2.3(0.3)	62.5	0.190(0.081)	0.358(0.075)
E. lateralis	Adults	18.1 (24)	2.3 (0.3)	71.4	0.158(0.078)	0.324 (0.075)
	Larvae	14.1 (21)	2.6(0.3)	87.5	0.207 (0 082)	0.454 (0.060)

TABLE IV - *Bootstrap mean estimates and confidence intervals (C. i.) for heterozygosities observed (H_o) and expected (H_e) under Hardy-Weinberg equilibrium in the three species of Heptageniidae.*

Species	Stage	H_o	C.i.	H_e	C.i.
Ecdyonurus sp.	Adults	0.1650	0 1600-0.1700	0.3660	0.3604-0.3716
	Larvae	0.2550	0.2481 -02619	04200	04146-04254
E. grandiae	Adults	0.2110	0.2104-0.2116	03340	03337-0.3343
	Larvae	0.2010	0.1948-0.2072	03790	03734-0.3846
E. lateralis	Adults	0.1470	0.1465-0 1475	0.3130	0.3126-03134
	Larvae	0.2170	02099-0.2241	04820	04779-0.4861

heterozygotes for the larvae, whereas in adults significant heterozygote deficiencies were found.

The results of the χ^2 contingency tests between both adult and larval samples of the two species of *Electrogena* are given (Table VI). Neither genotype nor allele frequencies of Adk-1, Ck-1, Gpi, Me-2, Pgm-1 differed significantly between the adults, whereas the Mdh-1 locus differed only for the genotype frequencies. In the larvae, the Ck-2 locus is fixed for the same genotype and allele in both species (Table II) and no test was performed; neither genotype nor allele frequencies of Adk-2, Gpi, La-1, Me-1, Me-2, Pap, Pgm-1 differed significantly between species, whereas only genotype frequencies did not vary significantly for La-2 and Mdh-1, and only allele frequencies did not vary significantly for Est-1, It is interesting to note that some loci differ markedly both in their frequencies and discriminatory power between species for adult and larval stages: for example, Pap differs significantly between the adults of the two species for the presence of the «a» allele in *E. grandiae*, while it is absent in *E. lateralis.* However, in the larvae it is the only allele found in *E. grandiae* and in most *E. lateralis*, thus being no longer diagnostic. Adults have more significantly variable loci (nine) than larvae (seven).

The χ^2 contingency tests for each species on both genotype and allele frequencies of the 11 loci shared by adults and larvae are given in Table VII. The Aat locus presented only one fixed allele and genotype both in larvae and adults of each species. In *Ecdyonurus* sp., genotype frequencies did not differ significantly in Est-2, La-2, Mpi, Pap and Pgm-1, whereas the only not significant allele frequencies were those of La-2 and Pgm-1. In *E. grandiae,* neither genotype nor allele frequencies of Est-2, Gpi, La-2, Mdh-1, and Pgm-1 differed between stages. In *E. lateralis,* Gpi, Mpi and Pgm-1 have genotype and allele frequencies that did not differ, whereas only allele frequencies at Mdh-1 did not differ significantly.

TABLE V - *Estimations of Hardy-Weinberg equilibrium and heterozygote excess for each polymorphic locus in adults and larvae of the three species of Heptageniidae. A, Pearson χ^2 goodness-of-fit test, with (n. genotypes-1) degrees of freedom (associated probability in brackets); B, χ^2 tests with pooling (when more than two alleles where observed), with 1 degree of freedom (associated probability in brackets); C, probabilities associated to the exact significance tests; F, fixation indices.*

Locus		Species					
		Ecdyonurus sp.		*E.grandiae*		*E. lateralis*	
		Adults	Larvae	Adults	Larvae	Adults	Larvae
Ada	A	41.825 (0.000)	12.802(0.046)	9.948 (0.002)	8.561 (0.036)		19.674 (0.000)
	B	15.158 (0.000)	6.697(0.010)		2.805 (0.094)		
	C	0.001	0.015		0.142		
	F	1.000	-0.406	0.757	-0 554		
Adk-1	A	1.286(0.257)		1.485 (0.223)		12.766 (0.000)	
	C	0.348		0.326		0.001	
	F	0.257		0.270		0.720	
Adk-2	A		9.143 (0002)		8,229 (0.004)		6.400 (0.011)
	C		0.111		0.012		0.048
	F		1.000		1.000		1.000
Ck-1	A	3.169 (0.075)		5.539 (0.019)		4.777 (0,039)	
	C	0.199		0.033		0.065	
	F	0.467		0.569		0.399	
Ck-2	A		5.333 (0.021)				
	C		0.086				
	F		1 000				
Est.l	A		0.298 (0.960)		46.598 (0.000)		25.596(0000)
	B		0.139 (0.709)		22.097 (0.000)		0.098(0.754)
	C		1.000		0.000		1.000
	F		0.018		1.000		0.041
Est-2	A	28.000 (0.002)	19.241 (0.037)	14.569 (0.002)	29.802 (0.001)	13.091 (0.000)	49.367 (0.000)
	B	2.286 (0.131)	3.316 (0.069)	2.310 (0 129)	12.299 (0.000)		2.848 (0.091)
	C	0.625	0.129	0.261	0.001	0.000	0.140
	F	0.744	-0.050	0.519	0.273	1.000	0.498
α-Gpdh	A			11.487 (0.001)		12.800 (0.005)	
	B					6.720 (0.010)	
	C			0.012		0.046	
	F			1.000		0.747	
Gpi	A	17.590 (0.001)	10.165 (0.017)	47.955 (0.000)	44.713 (0.000)	121.594 (0.000)	38.781 (0.000)
	B	7.535 (0.006)	9.219(0.002)	17.096 (0.000)	11.292 (0.001)	21.889 (0.000)	19.464 (0.000)
	C	0.021	0.005	0,000	0.001	0.000	0.000
	F	0.681	-0.648	0.751	0.695	0.795	0.859
La-1	A				7.619 (0.006)		7.200 (0.007)
	C				0.030		0.022
	F				1.000		1.000
La-2	A	5.333 (0.149)	14.667 (0.002)		9.143 (0.002)		7.200 (0.007)
	B	0.899 (0.346)	5.333 (0.021)				
	C	1.000	0.086				0.143
	F	0.455	1.000		1,000		1.000
Mdh-1	A	33.474 (0.000)	17.474 (0.008)	6.000 (0.014)	2.536 (0,469)	16.738 (0.010)	7.139 (0.308)
	B	5.540 (0.019)	6.955 (0.008)		0.768 (0.381)	9.657	0.889 (0.571)
	C	0.034	0.021	0.037	0.542	0.002	0.346
	F	-0.625	0.647	-1.000	-0.125	-0.543	0.092
Mdh-2	A	13.822 (0.003)					
	B	3.510(0.061)					
	C	0.099					
	F	0.569					
Mc-1	A		18.286(0.000)		12.121 (0.000)		52.418 (0.000)
	B						23.230 (0.000)
	C		0.000		0.001		0.000
	F		1.000		1.000		1.000
Mc-2	A	13.976 (0.000)		23.048 (0.000)		49.021 (0.000)	28.718 (0.000)
	C	0.002		0.043		0.020	0.002
	F	1.000		1.000		1.000	1.000
Mpi	A	22.095 (0.000)	9.333 (0.002)			10.159 (0.001)	30.413 (0.000)
	B	8.229 (0.004)					11.111 (0.001)
	C	0.012	0.007			0.003	0.001
	F	1.000	1.000			1.000	1.000
Pap	A		54.705 (0.000)	22.187 (0.000)		53.020 (0.000)	31.380 (0.000)
	B		19.352 (0.000)				
	C		0.000	0.000		0.019	0.001
	F		1.000	1.000		1.000	1.000
Pgm-1	A	1.089 (0.297)	0.487 (0.485)	14.429 (0.025)	6.889 (0.331)	23.476 (0.001)	7.030 (0.318)
	B			2.423 (0.120)	0.218 (0.641)	0.394 (0.530)	0.030 (0.862)
	C	0.420	0.573	0.255	0.001	1.000	1.000
	F	0.300	0.167	-0.379	-0,250	0.124	-0.189
Pgm-2	A						7.200 (0.007)
	C						0.143
	F						1.000

TABLE VI - *Pearson χ^2 tests on contingency tables of genotype and allele frequencies between the two* Electrogena *species at both adults and larval stages. g, number of genotypes; a, number of alleles.*

(Stage	Locus	Genotype frequencies				Allele frequencies			
		g	χ^2	d.f.	P	a	χ^2	d.f.	P
Adults	Aat	2	8.000	1	0.000	2	36.000	1	0.000
	Ada	3	33.600	2	0.000	2	63.344	1	0.000
	Adk-1	3	2.872	2	0.238	2	3.039	1	0.081
	Ck-1	3	0.450	2	0.799	2	0.233	1	0.629
	Est-2	5	1.566	4	0.000	4	33.974	3	0.000
	a-Gpdh	4	12.760	3	0.005	3	21.590	2	0.000
	Gpi	7	3.033	6	0.805	6	3.950	5	0.557
	Mdh-1	3	0.545	2	0.008	4	8.400	3	0.038
	Me-2	3	2.579	2	0.275	3	5.159	2	0.076
	Mpi	3	5.000	2	0.001	3	30.000	2	0.000
	Pap	3	2.783	2	0.002	3	25.566	2	0.000
	Pgm-1	6	7.143	5	0.210	4	0.486	3	0.922
	Pgm-2	2	0.000	1	0.003	2	18.000	1	0.000
	Totals		148.331	34	0.000		249.742	27	0.000
Larvae	Aat	2	18.000	1	0.000	2	36.000	1	0.000
	Ada	5	31.489	4	0.000	3	18.169	2	0.000
	Adk-2	2	0.010	1	0.921	2	0.020	1	0.889
	Est-1	5	20.000	4	0.000	3	1.315	2	0.518
	Est-2	11	24.966	10	0.005	5	27.097	4	0.000
	Gpi	6	5.227	5	0.389	4	2.156	3	0.541
	La-1	2	0.343	1	0558	2	0.686	1	0.408
	La-2	3	5.760	2	0.056	3	11.520	2	0.003
	Mdh-1	7	8.900	6	0.179	5	11.431	4	0.022
	Me-1	3	1.827	2	0.401	3	3.655	2	0 161
	Me-2	2	1.065	1	0.302	2	2.129	1	0.145
	Mpi	3	13388	2	0.001	3	26.775	2	0.000
	Pap	2	1.746	1	0 186	2	3.492	1	0.062
	Pgm-1	4	0.153	3	0.985	4	0.087	3	0.993
	Pgm-2	2	3.938	1	0.047	2	7.875	1	0.005
	Totals		136.812	44	0.000		152.407	30	0.000

Mean estimates of D, G_{ST}, and ϑ with their 99% confidence limits resulting from bootstrap resampling are given (Table VIII). For each species, the estimated values for adults are significantly higher than those for larvae, i.e., their confidence intervals do not overlap. Adult values of D, G_{ST}, and ϑ between the two *Electrogena* species and those between *Ecdyonurus* sp. and *E. lateralis* are very similar, whereas the same values in the larval array are significantly lower between the two *Electrogena* species than those between *Ecdyonurus* sp. and *E. lateralis.*

DISCUSSION

A number of electromorphs that differ from one stage to another in their presence/absence or in frequencies was found for each of the three species of Heptageniidae. Kownacki & Starmach (1984) observed electrophoretic patterns of non-specific esterases in larvae and adults of a number of species of Siphlonuridae, Heptageniidae and Ephemerellidae from Poland and did not find any difference between stages; on the contrary, our study detected the locus Est-1 that was exclusively expressed in the larvae, and locus Est-2 shared by both stages, whose genotype and allele frequencies both differed significantly between stages in *E. lateralis* and allele frequencies alone differed significantly in *Ecdyonurus* sp. Söderstrøm & Nilsson (1986) used starch-gel electrophoresis to compare electromorphs at the Lap locus in two Fennoscandian species of *Parameletus* (Siphlonuridae). They found different electromorphs between adults and nymphs, and only the latter were diagnostic between species. In the present study, two loci of Lap (= La) were found, with only one expressed by the adults; allele frequencies differed significantly between species only in the larvae (even if small samples were compared). The discrepancy between our results and those of the cited authors could be due either to species-specific interstage variation at the same locus, or to different experimental techniques.

Our study detected a higher number of presumptive loci in the larvae, even if a better discrimination between species was found in the adult array. Consequently, also the estimates of fixation and distance resulted in higher values between the adult samples; however, the distance values for the larvae gave relatively better discrimination between the two genera.

Zurwerra *et al.* (1987) analysed 16 electrophoretic loci in adults of 124 populations beloging to a number of European Heptageniidae, including the two species of *Electrogena* in our study. It is not easy to compare their results with ours, since they report only the most frequent allele found among all the populations of the

TABLE VII - *Pearson χ^2 tests on contingency tables of genotype and allele frequencies between adults and larvae of the three species of Heptageniidae. g, number of genotypes; a, number of alleles.*

Species	Locus	Genotype frequencies				Allele frequencies			
		g	χ^2	d.f.	P	a	χ^2	d.f.	P
Ecdyonurus sp.	Ada	7	31.000	6	0.000	4	10.086	3	0.018
	Est-2	10	13.778	9	0.130	7	14.959	6	0.021
	Gpi	6	28.225	5	0.000	4	9.750	3	0.021
	La-2	5	6.000	4	0.199	2	0.003	1	0.958
	Mdh-1	6	17.000	5	0.004	4	16.484	3	0.001
	Me-2	3	19.283	2	0.000	3	38.565	2	0.000
	Mpi	3	3.058	2	0.217	3	6.116	2	0.047
	Pap	3	5.373	2	0.068	3	10.747	2	0.005
	Pgm-1	3	0.486	2	0.784	2	0.471	1	0.493
	Pgm-2	2	6.000	1	0.014	2	12.000	2	0.000
	Totals		124.203	34	0.000		119.181	24	0.000
E. grandiae	Ada	5	22.223	4	0.000	3	15.920	2	0.000
	Est-2	7	10.649	6	0.100	5	6.900	4	0 141
	Gpi	6	2.543	5	0.770	5	2.366	4	0.669
	La-2	2	0.686	1	0.408	2	1.371	1	0.241
	Mdh-1	4	5.657	3	0.130	3	1.659	2	0.436
	Me-2	3	9.224	2	0.010	3	18.448	2	0.000
	Pap	2	18.129	1	0.000	2	36.259	1	0.000
	Pgm-1	4	3.010	3	0.390	4	1.396	3	0.706
	Pgm-2	2	7.000	1	0.008	2	14.000	1	0.000
	Totals		78.435	25	0.000		96.948	19	0.000
E. lateralis	Ada	4	52.000	3	0.000	3	38.397	2	0.000
	Est-2	7	15.572	6	0.016	5	25.208	4	0.000
	Gpi	7	3.937	6	0.685	5	3.059	4	0.548
	Mdh-1	7	9.977	6	0 126	5	2.411	4	0.661
	Me-2	4	27.721	5	0.000	4	55.443	3	0.000
	Mpi	3	0.950	2	0.622	3	1.900	2	0.387
	Pap	3	43.546	2	0.000	3	87.091	2	0.000
	Pgm-1	6	6.310	5	0.277	4	0.984	3	0.805
	Pgm-2	2	5.625	1	0.018	2	11.250	1	0.001
	Totals		165.638	36	0.000		225.743	25	0.000

TABLE VIII - *Estimations of parameters of genetic divergence between the three species of Heptageniidae at both adult and larval stages. Values in the Global columns are computed from the global set of loci, whereas those in the Mean columns are mean values among 1000 bootstrap repetitions over loci, with related confidence intervals in brackets. P, Parameter; D, Nei's unbiased standard distance; G_{ST}, Nei's unbiased G_{ST}; ϑ, Weir-Cockerham's unbiased ϑ.*

p	OTUs		Adults			Larvae		
			Global	Mean	C.i.	Global	Mean	C.i.
D	*Ecdyonurus* sp.	*E. grandiae*	0.974	1.024	1.001-1.047	0.743	0.811	0.788-0.834
	Ecdyonurus sp.	*E. lateralis*	0.659	0.758	0.730-0.771	0.465	0.478	0.465-0.491
	E. grandiae	*E. lateralis*	0.608	0.669	0.646-0.688	0.342	0.398	0.384-0.413
G_{ST}	*Ecdyonurus* sp.	*E. grandiae*	0.363	0.371	0.365-0.378	0.287	0.289	0.283-0.296
	Ecdyonurus sp.	*E. lateralis*	0.313	0.345	0.338-0.351	0.191	0.188	0.183-0.192
	E. grandiae	*E. lateralis*	0.317	0.345	0.336-0.354	0.174	0.176	0.170-0.182
ϑ	*Ecdyonurus* sp.	*E. grandiae*	0.533	0.535	0.528-0.541	0.446	0.449	0.441-0.457
	Ecdyonurus sp.	*E. lateralis*	0.477	0.505	0.498-0.512	0.320	0.318	0.311-0.324
	E. grandiae	*E. lateralis*	0.482	0.507	0.497-0.517	0.296	0.301	0.293-0.310

same species. The value of genetic identity (I = 0.43) between the two species reported by them is lower than ours (I = e^{-D} = 0.54). Zurwerra *et al.* (1987) reported only mean identity values between taxa, which resulted from the pooling of all the populations of the same species, and they did not give information about the dispersion of the data around means; thus, we do not know if our values fall within the ranges around their mean identity values. Also in this case, results that differ between studies could arise from differences in the experimental techniques and also in the examined loci. However, our results agree with an underlying closer relationship between the two *Electrogena*, when compared to the *Ecdyonurus* species.

According to Thorpe (1982), a good positive correlation is generally found between genetic identity/distance and systematic level, an indirect proof of the existence of the so-called molecular clock. A number of authors use values of genetic identity to decide about the systematic rank of different populations (e.g., Funk *et al.*, 1988; Hefti *et al.*, 1988, 1989; Savolainen *et al.*, 1991; Sweeney & Funk, 1991; Belfiore *et al.*, 1992; Studemann *et al.*, 1994). Our results suggest caution in the use of absolute values of identity, since different conclusions could be drawn for the same pair of populations depending on which developmental stage is examined.

Most of the electrophoretic systematic studies limit comparisons to allele frequencies, with no mention about genotypes: this is acceptable only if populations are in H-W equilibrium (e.g., Weir, 1990). On the contrary, our data indicate that for a number of loci the hypothesis of H-W equilibrium is not supported (even if this can be a consequence of casual deviations in some loci in which small samples were considered), with differences between stages being sometimes species-specific: in any case, genotype frequencies should always be given in this kind of study, or at least heterozygosities should be presented. The available studies, which provide information about fixation indices (Sweeney *et al.*, 1986, 1987; Sweeney & Funk, 1991) report F_{ST} values for intraspecific comparisons sensibly lower than those obtained by us between species and genera for both developmental stages. The observed heterozygosity values for adults are in accordance with those reported by Powell (1975) for Insecta and by Studemann *et al.* (1994) for Ephemeroptera. It is worth noting, however, that the observed heterozygosities in our sample, even though they differed between stages, resulted in values lower than those expected under H-W equilibrium, with only one exception. Such heterozygote deficiencies are also found in a number of North American species (Sweeney *etal.*, 1987; Sweeney & Funk, 1991). These findings can be explained by an inbreeding effect (Sweeney *et al.*, 1987), or by historical events leading to genetic bottlenecks and/or founder effects (Sweeney & Funk, 1991). More data from a number of conspecific populations and species are needed to support one or another of the cited hypotheses for the species examined in the present paper; however, we observed a large number of individuals per population at our collecting sites, which gives more credence to the historical events hypothesis. Similarly, we cannot offer an explanation for the higher levels of heterozygosity in the larvae. This is a rather unusual finding, since these higher levels should be observed in the adults, or in the stages with higher dispersal capabilities (e.g., Kalezic & Tucic, 1984).

According to the neutral theory of molecular evolution, the rate of evolution at the molecular level is roughly the same for a particular gene or set of genes, and the rate of molecular divergence among lineages is approximately constant (e.g., Wilson *et al.*, 1977; Kimura, 1983; Nei, 1987; Moritz & Hillis, 1990). Our findings seem to conflict with this theory, since the differences found between stages in the same species would indicate that the rate of molecular divergence changes according to the developmental stage. Some caution, however, should be taken. The criterion for assessing homologies in an electrophoretic comparison of proteins is based on mobility and specificity for a given substrate, not on direct observations of peptide or DNA sequences. Thus, one cannot be absolutely sure that a system of electromorphs observed in a given developmental stage is homologous to the same system found in another stage, even if in the same population. Indeed, our work as well as those of Basha & Pescador (1984) and Kownacki & Starmach (1984) have demonstrated that a large number of loci (about 50% of the total in our data) are expressed in only one stage. As a consequence, rates of molecular divergence differ between developmental stages since the electromorphs represent products of different genes. Thus, to prevent incorrect assessments of homology and a considerable loss of useful loci, systematic comparisons at the molecular level should be based on the same stage in all the considered taxa (see also Matha & Šula, 1984).

The principal conclusion that can be drawn from the present study is that electrophoretic comparisons between populations using different developmental stages should be conducted carefully or avoided, if possible. When comparing individuals at the same developmental

stage, similar results seem to be obtained from within-stage comparisons, but the absolute values of a number of parameters (such as estimations of H-W equilibrium, heterozygosities, fixation and gene flow, identities and distances) are different, and care should be taken in drawing conclusions based on these values.

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