

**GENETIC DIVERGENCE BETWEEN TWO INSULAR SPECIES
OF THE GENUS *RHITHROGENA*
(Ephemeroptera, Heptageniidae) (*)**

CARLO BELFIORE, ORFEO PICARIELLO, GIOVANNI SCILLITANI,
MASSIMO CRETELLA(**)

Ephemeroptera are one of the most abundant and common components of running water benthic communities. A well established taxonomy of this group is therefore priority to studies and quality monitoring of rivers. Phylogenetical and biogeographical studies must be also supported by the ability of recognizing and naming species. In fact, various problems (see Belfiore & D'Antonio, in press) prevented an adequate development of the taxonomy of European mayflies. The classical morphology analysis is in most cases insufficient to clear up the status of many taxa, with regard to the difficult identification of diagnostic characters and to the problems of variability between populations. The biosystematic approach, using methods from heterogeneous disciplines, often helped both to characterize closely related species and to cluster them at a supraspecific level. The biochemical methods, recently applied also to the Ephemeroptera taxonomy (Zurwerra et al., 1982; 1984; 1985; 1986; 1987), have proven to be a very useful tool in several cases of uncertain discrimination of taxa. Therefore we are carrying a study on most problematic taxa, for obtaining biochemical data complementary to the other morphological analyses.

Electrophoresis of proteins is a quick method for a genetic characterization of populations. Notwithstanding the wide application of biochemical systematics' to a lot of taxonomic groups (e.g.: Nei, 1987), Ephemeroptera were only recently tested: methodological calibration must be therefore the first step in this study; other

(*) Research carried out with MPI grant (40% and 60%).

(**) Dipartimento di Zoologia dell'Università di Napoli "Federico II", Via Mezzocannone, 8 - 80134 Napoli.

relevant problems concern the use of the different developmental stages as study material, the diagnosis of closely related species, the characterization of species-group, the phylogenetic reconstruction with a calibration of the molecular clock.

In this first contribution we deal with the methodological calibration and with the diagnosis of two close species of the genus *Rhithrogena*.

The genus *Rhithrogena* includes many species, most of which recently described, which are frequently matter of question for the specialists. The available characters shared by the adults are few and of poor diagnostic value; larvae from different populations are widely variable. Also classification at species-group level is often hard, because of the incongruence between larval and imaginal characters. In this paper we study two insular taxa: *Rhithrogena insularis* Esben-Petersen 1913, endemic to Corsica, and *R. nuragica* Belfiore, 1987, endemic to Sardinia. All developmental stages of both species are adequately described; in spite of this fact, their specific status is still unclear. The shape of male genitalia is to some extent variable within populations, the general arrangement being similar in both species (see Belfiore, 1987: figs. 1-2). Larvae have hardly appreciable differences, with a wide variability within and among the populations. Considering the whole set of characters, both species are considerably different from all the continental representatives of the genus. Some features (crenulated margin of gills, tore margin of lateral sclerite on I abdominal sternites perpendicular to body axis: Belfiore, 1987, figs. 31-32; 19-20) relate the larvae to the *hybrida*-group; other characters (triangular plica on the first gill, reddish spot on femora: Belfiore, 1987, figs. 31-32; 28-29) are *semicolorata*-group-like. On the other hand, eggs (Belfiore, 1987: figs. 15-18) are surprisingly different from all the other species of *Rhithrogena* and are also the only character useful for a sure specific determination, together with the collection site. Chorion lacks knob-terminated coiled threads, and has characteristic finger like projections, small and subconical in *R. nuragica*, largest, and with tree-like ramifications, in *R. insularis*.

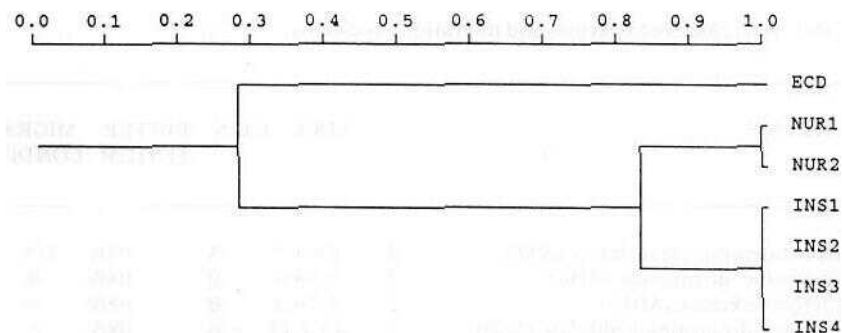


Fig. 1 — UPGMA dendrogram constructed on the basis of Nei's identity indices. (ECD - *E. corsicus*; NUR 1-2 - *R. nuragica*; INS 1-4 - *R. insularis*).

MATERIAL AND METHODS

We studied two populations of *R. nuragica* (Sardinia: NUR1, Desulo (NU), Rio Aratu, 4.VI.1989; NUR2, Gennargentu, Arcu Guddetorgiu, right trib. of Flumendosa River, 4.VI.1989) and four of *R. insularis* (Corsica: INS1, Ghisoni, Fium'Orbo, 9.VI.1989; INS2: D 368 between Zona-Porto Vecchio, L'Ospedale, 11.VI.1989; INS3, Corte, Restonica River, 7.VI.1989; INS4, Haut Asco, Stranciacone River, 8.VI.1989). Seven male imagines per population were analysed; moreover, we analysed four male imagines of *Ecdyonurus corsicus* Esben-Petersen, 1912 (Sardinia: ECD, Road SS 389, Km 157 (NU), Rio Calaresu, 5.VI.1989) for comparison. Each specimen was homogenized in toto in distilled water (1:1 v/w), and the liquid fraction was adsorbed on paper pieces (Macherey-Nagel 1640 m) and then applied on 10% starch gel (Sigma potato starch S4501); proteins were separated by horizontal electrophoresis. The enzymes assayed, the buffers employed and the migration conditions are listed in table 1. The electromorphs were revealed by standard histo-chemical techniques (Shaw and Prasad, 1970; Selander et al., 1971; Ayala et al., 1972). Each system of electromorphs was interpreted as a product of a single presumptive locus; the isozyme loci were designated by numbers, with «1» being the least rapidly anodal migrating. The different electromorphs in a single system were interpreted as alternative forms encoded by alleles of a single locus, and designated by small letters, where «a» is the least rapidly anodal migrating. From the electrophoretic data the allelic frequen-

Table 1 — Assayed enzymes and migration conditions.

| ENZYME | | LOCI | E.C.N. | BUFFER | MIGRATION SYSTEM | CONDITIONS |
|--|---|----------|--------|--------|------------------|------------|
| Aspartate aminotransferase (AAT) | 2 | 2.6.1.1 | A | 150V, | 17h | |
| Adenosine deaminase (ADA) | 1 | 3.5.4.4 | B | 100V, | 4h | |
| Adenylate kinase (ADK) | 2 | 2.7.4.3 | B | 100V, | 5h | |
| Fructose-diphosphate aldolase (ALD) | 1 | 4.1.2.13 | B | 100V, | 5h | |
| Aldehyde dehydrogenase (ALDH) | 1 | 1.2.1.3 | C | 60V, | 16h | |
| Creatine kinase (CK) | 1 | 2.7.3.2 | B | 100V, | 5h | |
| Carboxylesterase (EST) | 2 | 3.1.1.1 | A | 75V, | 15h | |
| Fructose-diphosphatase (FDPH) | 2 | 3.1.3.11 | B | 100V, | 6h | |
| Glutammate dehydrogenase (GDH) | 1 | 1.4.1.2 | C | 60V, | 15h | |
| Glycerol-3-phosphate dehydrogenase (aGPDH) | 1 | 1.1.1.8 | B | 100V, | 5h | |
| Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) | 1 | 1.2.1.12 | B | 100V, | 5h | |
| Glucose-phosphate isomerase (GPI) | 1 | 5.3.1.9 | B | 100V, | 5h | |
| Hexokinase (HK) | 2 | 2.7.1.1 | B | 100V, | 4h | |
| Isocitrate dehydrogenase (NADPdep.) (IDH) | 2 | 1.1.1.42 | B | 100V, | 7h | |
| Leucine amino-peptidase (LAP) | 1 | 3.4.11.1 | A | 90V, | 19h | |
| Lactate dehydrogenase (LDH) | 1 | 1.1.1.27 | D | 80V, | 14h | |
| Malate dehydrogenase (NADdep.) (MDH) | 2 | 1.1.1.37 | B | 100V, | 5h | |
| Malate dehydrogenase (NADPdep.) (ME) | 1 | 1.1.1.40 | B | 100V, | 5h | |
| Mannose-phosphate isomerase (MPI) | 1 | 5.3.1.8 | B | 100V, | 4h | |
| Phenylalanyl-proline peptidase (PAP) | 1 | 3.4.1.1 | A | 90V, | 19h | |
| Phosphoglucosmutase (PGM) | 1 | 2.7.5.1 | D | 80V, | 14h | |
| Superoxide dismutase (SOD) | 2 | 1.15.1.1 | B | 100V, | 5h | |

A - Discontinuous Tris-citrate, pH 8.2 + lithium borate, pH 8.1 (Selander et al., 1971).

B = Continuous Tris-citrate, pH 8.0 (Shaw & Prasad, 1970).

C=Continuous Tris-citrate, pH 7.0 (A y a l a & a l ., 1972).

D - Tris-maleate-EDTA, pH 7.4 (Shaw & Prasad, 1970).

cies were computed; average heterozygosities corrected for sample Sizes and indices of genetic identity and unbiased distance (Nei, 1978) were computed from the allelic frequencies. Significance of identity values was assessed by a χ^2 test for gene frequencies (Nei, 1987). An UPGMA dendrogram (Sneath & Sokal, 1973) was built by the genetic identities.

RESULTS

The electrophoretic analysis detected 22 enzymes for a total of 30 presumptive loci (table I): five of those (Ald, Aldh, Gdh, Ldh and Mdh-2) were monomorphic and fixed for the same allele. Five loci (Est-1, Est-2, Gpi, Lap and Pgm) were diagnostic between *R. nuragica* and *R. insularis*. The allelic frequencies at variable loci are in table 2. Average heterozygosities along with Nei's indices of genetic identity and unbiased distance are listed in table 3. The mean genetic identity between *R. nuragica* and *R. insularis* was 0.820, which significantly differs from 1. Intraspecific variability in both species was not detected. The distances of both species from *E. corsicus* are alike. Mean heterozygosities are similar in all the populations. The dendrogram (fig. 1) shows three clusters corresponding to the three species.

DISCUSSION

Genetic data, specially distance and identity estimates, are subjected to stochastic errors which affect the reliability of results. Several authors (German & Renzi, 1979; Nei, 1978; 1987) suggest to sample a high number of genetic loci to prevent this problem. The present work assayed a higher number of loci (30) than previous papers on electrophoresis of Ephemeroptera (16-17 loci: Zurwerra et al., 1982; 1984; 1985; 1986; 1987).

Several authors (i.e.: Thorpe, 1982; Nei, 1987) found a high empirical positive correlation between genetic divergence and taxonomic level. Thorpe (1982) suggests that the index of genetic identity can be used to state the taxonomic rank of doubtful allopatric populations, in lack of clear evidences from morphology, physiology, ethology, etc. This author states that the taxa whose identity index is less than 0.85 can be considered as valid species.

The very low divergence found between conspecific populations of *R. nuragica* and *R. insularis* contrasts with the variability found at morphological level, as sometime has been found in other groups (e.g.: Lewontin, 1984; Allegrucci et al., 1987). On the other hand, the live diagnostic loci indicate a relevant genetic divergence between the two species. These data, in accordance with the egg differences, confirm the specific validity of the two taxa.

Table 2 — Allelic frequencies at variable and diagnostic loci.

| LOCUS | ALL | NUR1 | NUR2 | INS1 | INS2 | INS3 | INS4 | ECD |
|---------------|-----|-------|-------|-------|-------|-------|-------|-------|
| Aat-1 | a | | | | | | | 1.000 |
| | b | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| Aat-2 | a | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| | b | — | — | — | — | — | — | 0.500 |
| | c | — | — | — | — | — | — | 0.500 |
| Ada | a | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| | b | — | — | — | — | — | — | 1.000 |
| Adk-1 | a | — | — | — | — | — | — | 1.000 |
| | b | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| Adk-2 | a | — | — | — | — | — | — | 0.750 |
| | b | — | — | — | — | — | — | 0.250 |
| | c | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | — |
| | d | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | — |
| Ck | a | — | — | — | — | — | — | 0.875 |
| | b | — | — | — | — | — | — | 0.125 |
| | c | 0.500 | 0.571 | 0.500 | 0.500 | 0.500 | 0.500 | — |
| | d | 0.500 | 0.429 | 0.500 | 0.500 | 0.500 | 0.500 | — |
| Est-1 | a | 1.000 | 1.000 | — | — | — | — | — |
| | b | — | — | 0.857 | 0.928 | 0.643 | 0.714 | — |
| | c | — | — | 0.143 | 0.071 | 0.357 | 0.286 | — |
| | d | — | — | — | — | — | — | 0.500 |
| | e | — | — | — | — | — | — | 0.500 |
| Est-2 | a | 1.000 | 1.000 | — | — | — | — | — |
| | b | — | — | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| Fdph-1 | a | — | — | — | — | — | — | 1.000 |
| | b | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| Fdph-2 | a | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 |
| | b | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 |
| α Gpdh | a | — | — | — | — | — | — | 1.000 |
| | b | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| G3pdh | a | — | — | — | — | — | — | 1.000 |
| | b | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| Gpi | a | 1.000 | 1.000 | — | — | — | — | — |
| | b | — | — | — | — | — | — | 1.000 |
| | c | — | — | 1.000 | 1.000 | 1.000 | 1.000 | — |
| Hk-1 | a | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| | b | — | — | — | — | — | — | 1.000 |
| Hk-2 | a | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| | b | — | — | — | — | — | — | 1.000 |
| Idh-1 | a | — | — | — | — | — | — | 1.000 |
| | b | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| Idh-2 | a | — | — | — | — | — | — | 1.000 |
| | b | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | — |
| | e | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | — |

| | | | | | | | | |
|-------|---|-------|-------|-------|-------|-------|-------|-------|
| Lap | a | | | 1.000 | 1.000 | 1.000 | 1.000 | |
| | b | 1.000 | 1.000 | | | | | 0.500 |
| | c | | | | | | | 0.500 |
| Mdh-1 | a | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 |
| | b | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 |
| Me | a | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | — |
| | b | — | | | — | | — | 1.000 |
| Mpi | a | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | |
| | b | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | |
| | c | — | — | — | — | — | — | 1.000 |
| Pap | a | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | |
| | b | | | | | | | 1.000 |
| Pgm | a | | | 0.071 | | 0.071 | | |
| | b | 1.000 | 1.000 | — | | — | | 0.500 |
| | c | | | 0.928 | 1.000 | 0.857 | 1.000 | 0.500 |
| | d | | | — | | 0.071 | | — |
| Sod-1 | a | | | — | | — | | 1.000 |
| | b | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | |
| Sod-2 | a | | | | | | | 1.000 |
| | b | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | |

If further studies on Ephemeroptera will evidence a low genetic intraspecific variability versus a high divergence between species, electrophoresis can provide a reliable instrument for solving classification problems.

Table 3 — Indices of Nei's genetic identity (above diagonal), Nei's unbiased distance (below diagonal), and average heterozygosity (on diagonal).

| | NUR1 | NUR2 | INS1 | INS2 | INS3 | INS4 | ECD |
|-------|---------|---------|---------|---------|---------|---------|---------|
| NUR 1 | (0.108) | 1.000 | 0.821 | 0.817 | 0.826 | 0.821 | 0.262 |
| NUR2 | 0.000 | (0.108) | 0.820 | 0.817 | 0.826 | 0.821 | 0.262 |
| INS1 | 0.189 | 0.189 | (0.121) | 1.000 | 0.998 | 0.999 | 0.282 |
| INS2 | 0.194 | 0.194 | 0.000 | (0.112) | 0.996 | 0.998 | 0.282 |
| INS3 | 0.182 | 0.182 | 0.000 | 0.000 | (0.133) | 0.999 | 0.282 |
| INS4 | 0.188 | 0.188 | 0.000 | 0.000 | 0.000 | (0.122) | 0.283 |
| ECD | 1.325 | 1.325 | 1.253 | 1.253 | 1.251 | 1.247 | (0.129) |

SUMMARY

Some populations of *Rhithrogena nuragica* Belfiore and *R. insularis* Esb.-Pet., two species of uncertain taxonomic status, were investigated by electrophoresis of proteins. 22 enzymes were detected for 30 loci: five loci resulted monomorphic and five diagnostic between the *Rhithrogena* species. The mean genetic identity between *R. nuragica* and *R. insularis* was 0.820. The very low divergence between conspecific

populations and the differences between species, together with morphological data (e.g.: egg chorion), confirm the specific status of the taxa.

RIASSUNTO

Sono state studiate, mediante elettroforesi delle proteine su gel d'amido, alcune popolazioni di due specie il cui status tassonomico non era stato definitivamente chiarito: *Rhithrogena nuragica* Belfiore e *R. insularis* Esb.-Pet., endemiche rispettivamente della Sardegna e della Corsica. Sono stati evidenziati 22 sistemi enzimatici per un totale di 30 loci. Di questi, cinque sono risultati monomorfici e cinque diagnostici tra le due specie. Il valore di identità media tra le specie è risultato 0.820. La variabilità intraspecifica si è rivelata pressoché nulla. I dati confermano lo status specifico di *R. insularis* e *R. nuragica*.

REFERENCES

- ALLEGRUCCI, G., D. CESARONI & V. SBORDONI. 1987. Adaptation and speciation of *Dolichopoda* cave crickets (Orthoptera, Rhaphidophoridae): geographic variation of morphometric indices and allozyme frequencies. *Biol. Jour. Linn. Soc.*, 31: 154-160.
- AYALA, F.J., J.R. POWELL, M.L. TRACEY, C.A. MOURAO, S. PEREZ-SALAS. 1972. Enzyme variation in the *Drosophila willistoni* group. IV. Genic variation in natural populations of *Drosophila willistoni*. *Genetics*, 70: 113-139.
- BELFIORE, C. 1987. Heptageniidae from Corsica and Sardinia. *Rhithrogena nuragica* n. sp., *R. eatoni* Esben-Petersen 1912, and *R. insularis* Esben-Petersen 1913 (Ephemeroptera). *Annls Limnol.*, 23 (2): 87-94.
- BELFIORE, C. & C. D'ANTONIO. in press. Faunistic, taxonomic and biogeographical studies of Ephemeroptera from Southern Italy. *Proceedings of the VI Int. Conf. on Ephemeroptera*. Granada, 1989.
- GORMAN, G.C. & J. RENZI. 1979. Genetic distance and heterozygosity estimates in electrophoretic studies: effects of sample size. *Copeia*, 1979: 242-249.
- LEWONTIN, R.C. 1984. Detecting population differences in quantitative characters as opposed to gene frequencies. *The American Naturalist*, 123: 115-124.
- NEI, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89: 583-590.
- NEI, M. 1987. *Molecular evolutionary genetics*. Columbia Univ. Press, New York, 512 pp.
- SHAW, C.R. & R. PRASAD. 1970. Starch gel electrophoresis of enzymes— A compilation of recipes. *Biochem. Genet.*, 4: 297-320.
- SELANDER, R.K., M.H. SMITH, S.Y. YANG, W.E. JOHNSON, J.B. GENTRY. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old field mouse (*Peromyscus polionotus*). *Stud. Genet. VI, Univ. Texas Publ.*, 7103: 49-90.
- SNEATH, P.H.A. & R.R. SOKAL. 1973. *Numerical taxonomy*. Freeman, S. Francisco. 573 pp.
- THORPE, J.P. 1982. The molecular clock hypothesis: Biochemical evolution, genetic differentiation and systematics. *Ann. Rev. Ecol. Syst.*, 13: 139-168.
- ZURWERRA, A., I. TOMKA. 1982. Enzymeelektrophoretische Untersuchungen an der Gattung *Epeorus* Eaton (Heptageniidae, Ephemeroptera). *Mitt. Schweiz Entomol. Ges.*, 55: 359-360.
- ZURWERRA, A. & I. TOMKA. 1985. *Electrogena* gen. nov., eine neue Gattung der Heptageniidae (Ephemeroptera). *Ent. Berichte Luzern*, 13: 99-104.
- ZURWERRA, A., I. TOMKA & G. LAMPEL. 1984. Application of the scanning electron microscope and the enzyme-gel-electrophoresis to solve taxonomical problems: the European species of the genus *Epeorus* sensu Tshernova (1981) (Ephemeroptera, Heptageniidae). *Proc. IV Int. Conf. Ephemeroptera*, Bechyne: 213-218.
- ZURWERRA, A., I. TOMKA & G. LAMPEL. 1986. Morphological and enzyme electrophoretic studies on the relationships of the European *Epeorus* species (Ephemeroptera, Heptageniidae). *Syst. Entomol.*, 11: 255-266.
- ZURWERRA, A., M. METZLER & I. TOMKA. 1987. Biochemical systematics and evolution of the European Heptageniidae (Ephemeroptera). *Arch. Hydrobiol.*, 109: 481-510.