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ELECTROPHORETIC ANALYSIS OF *RANA LATASTEI* POPULATIONS (AMPHIBIA: RANIDAE) FROM ITALY AND ISTRIA (NW YUGOSLAVIA)

ANALISI ELETTROFORETICA DI POPOLAZIONI ITALIANE E IUGOSLAVE DI RANA LATASTEI *(AMPHIBIA: RANIDAE)*

Abstract – Allozyme variation in *Rana latastei* populations from N Italy and NW Yugoslavia (Istria) was studied by means of horizontal starch gel electrophoresis at 20 enzyme loci. Low levels of genetic differentiation were found comparing Italian and Istrian populations (Nei's average $D = 0.030$). The estimated time of divergence between the Yugoslavian and the Italian populations seems to go back to the Upper Pleistocene-Holocene. This is in agreement with the biogeographic hypotheses proposed by some authors, and testifies to the recent geographic isolation of the *Rana latastei* populations occurring in NW Yugoslavia.

Key words: *Rana latastei*, Amphibia, Multilocus electrophoresis, Allozyme variation.

Riassunto breve – *Vengono presentati i risultati di uno studio condotto mediante analisi elettroforetica di 20 loci enzimatici su alcune popolazioni di* Rana latastei *dell'Italia settentrionale (Pianura Padana) e della Iugoslavia nord-occidentale (Istria). I valori di distanza genetica di Nei osservati confrontando le popolazioni di* Rana latastei *dell'Istria e della Pianura Padana risultano essere relativamente bassi (*D *media = 0.030). La stima del tempo di divergenza evolutiva, ottenuta utilizzando la formula proposta da* NEI *(1975), indica che l'isolamento geografico delle popolazioni istriane di* R. latastei *risale probabilmente al tardo Pleistocene-Olocene. Tale evidenza è in accordo con le ipotesi biogeografiche e geologiche proposte da differenti autori.*

Parole chiave: Rana latastei, *Amphibia, Elettroforesi multilocus, Differenziamento genetico.*

Introduction

Rana latastei BOULENGER, 1879 is a brown frog endemic to Northern Italy, where it occurs with fragmented populations in the Padano-Veneta Plain wet broad-

leaved woods (POZZI, 1980; LANZA, 1983; DOLCE et al., 1985). Out of the geopolitical Italian boundaries the species has been so far recorded only in few localities of southern Switzerland (Mendrisiotto, Canton Ticino; GROSSENBACHER, 1982) and NW Yugoslavia (Istria: CEI, 1944; SCHMIDTLER, 1977; BURLIN & DOLCE, 1986). *R. latastei* does not occur in the Karst area, and the populations from eastern Padano-Veneta Plain are separated from the Istrian ones by a gap of ≈70 km (fig. 1).

Rana latastei specimens from Istria are morphologically very similar to the Italian ones (SCHMIDTLER, 1977). Since it is well known that in several Amphibians the patterns of morphometric and genetic variation are independent (see LANZA et al., 1984; CAPULA et al., 1985; NASCETTI et al., 1988), we can not exclude that the Istrian populations of *Rana latastei* are genetically differentiated from the Italian ones, but so far any genetic investigation has been carried out on the former populations.

BURLIN & DOLCE (1986) have hypothesized that the Yugoslavian populations of *R. latastei* would be geographically separated from the Italian ones about 8.000-10.000 years ago. According to these authors *R. latastei* colonized NW Yugoslavia from NE Italy during Upper Pleistocene (Würm), when the N Adriatic sea channel closed and a land bridge linked the plain of the Po river to the Istrian Peninsula (see MOSETTI & D'AMBROSI, 1967; D'AMBROSI, 1969, 1976).

In the present paper we have tested the hypothesis of the recent geographic isolation of the Italian and Yugoslavian *Rana latastei* populations studying their genetic variation with multilocus electrophoresis. The level of genetic divergence between the studied populations has been considered as an indirect indication of their isolation, and the time passed since the geographic isolation has been inferred from the genetic distance data using NEI's formula (1975).

Materials and Methods

A total of 35 specimens of *Rana latastei* from 5 localities was sampled to study intraspecific allozyme variation. Geographic origin and sample designations, together with the number of specimens analyzed per population, are as follows:

- Bosco del Merlino, Caramagna (Cuneo, Piedmont, NW Italy) (RLC), 5 specimens;
- Lago di Alserio, Merone (Como, Lombardy, N Italy) (RLM), 6 specimens;
- Punte Alberete (Ravenna, Emilia-Romagna, NE Italy) (RLR), 8 specimens;
- Bosco Baredi, Muzzana del Turgnano (Udine, Friuli, NE Italy) (RLT), 9 specimens;
- Oprtalj (Istria, NW Yugoslavia)(RLO), 7 specimens.

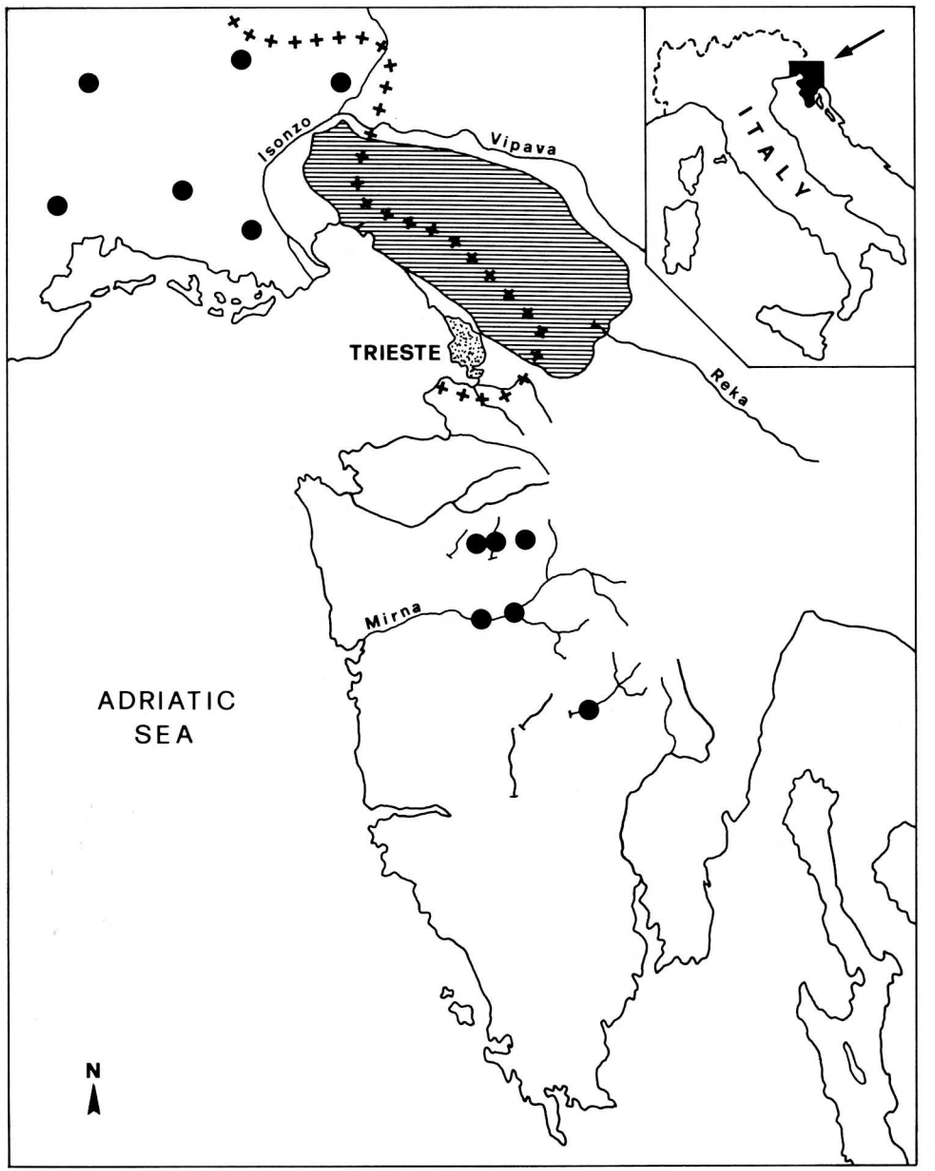

Fig. 1 - Present distribution of *Rana latastei* in NE Italy (Friuli) and NW Yugoslavia (Istria). The dashed area indicates the Karst region.
- *Distribuzione di* Rana latastei *nell'Italia nord-orientale (Friuli) e nella Iugoslavia nord-occidentale (Istria). L'area tratteggiata indica il Carso.*

Buffer system	Electrodes	Gel
1 Discontinuous Tris/citrate (Na) (POULIK, 1957)	0.3 M sodium borate, pH 8.2 (18.55 g boric acid, 2.40 g NaOH)	0.076 M Tris/0.005 M citric acid, pH 8.7 (9.21 g Tris, 1.05 g monohydrate citric acid)
2 Continuous Tris/citrate (SELANDER et al., 1971)	0.687 M Tris/0.157 M citric acid, pH 8 (83.2 g Tris, 30 g monohydrate citric acid)	0.023 M Tris/0.005 M citric acid, pH 8 (2.77 g Tris, 1.10 g monohydrate citric acid)
3 Tris/versene borate (BREWER & SING, 1970)	0.21 M Tris/0.15 M boric acid/0.006 M EDTA, pH 8 (25.4 g Tris, 9.24 g boric acid, 2.20 g EDTA)	0.021 M Tris/0.02 M boric acid/0.007 M EDTA, pH 8 (2.5 g Tris, 1.24 g boric acid, 0.25 g EDTA)
4 Phosphate/citrate (HARRIS, 1966)	0.15 M tri-sodium citrate/ 0.24 M sodium hydrogen phosphate, pH 6.3 (44.11 g sodium citrate, 33.12 g NaH_2PO_4)	electrode buffer diluted 1:40, pH 6.3
5 Tris/maleate (modified from BREWER & SING, 1970)	0.01 M Tris/0.1 M maleic acid/0.01 M EDTA/ 0.015 $MgCl_2$/0.125 M NaOH, pH 7.2 (12.11 g Tris, 11.61 g maleic acid, 3.72 g EDTA, 2.03 g $MgCl_2$, 5 g NaOH)	electrode buffer diluted 1:10, pH 7.4

Tab. I - Buffer systems (analytical grade reagents per litre; pH at room temperature).
- *Sistemi tampone.*

Enzyme	Buffer system	V/cm	Time (h)	References
Glycerol-3-phosphate dehydrogenase *aGPDH* (EC 1.1.1.8)	4	8	6	AYALA et al., 1972
Lactate dehydrogenase *LDH* (EC 1.1.1.27)	5	7	6	BREWER & SING, 1970
Malate dehydrogenase *MDH* (EC 1.1.1.37)	4	8	5	SHAW & PRASAD, 1970
Malic enzyme *ME* (EC 1.1.1.40)	2	8	5	AYALA et al., 1972
Isocitrate dehydrogenase *IDH* (EC 1.1.1.42)	2	8	5	SHAW & PRASAD, 1970
Phosphogluconate dehydrogenase *6PGD* (EC 1.1.1.44)	4	8	5	SHAW & PRASAD, 1970
Glyceraldehyde-phosphate dehydrogenase *G3PDH* (EC 1.2.1.12)	2	7	5	AYALA et al., 1972
Superoxide dismutase *SOD* (EC 1.15.1.1)	3	8	5	SELANDER et al., 1971
Purine nucleoside phosphorylase *NP* (EC 2.4.2.1)	3	8	4	HARRIS & HOPKINSON, 1976
Glutamate-oxaloacetate transaminase *GOT* (EC 2.6.1.1)	1 or 4	9	5	SELANDER et al., 1971
Creatine kinase *CK* (EC 2.7.3.2)	2	8	5	AYALA et al., 1972
Adenilate kinase *ADK* (EC 2.7.4.3)	2	8	5	AYALA et al., 1972
Phosphoglucomutase *PGM* (EC 2.7.5.1)	5	8	5	BREWER & SING, 1970
Adenosine deaminase *ADA* (EC 3.5.4.4)	2	8	5	HARRIS & HOPKINSON, 1976
Mannose phosphate isomerase *MPI* (EC 5.3.1.8)	3	8	4	HARRIS & HOPKINSON, 1976

Tab. II - Enzymes studied and electrophoretic procedures. Enzymes are arranged by Enzyme Commission Number (EC).
- *Enzimi studiati e procedure elettroforetiche. Gli enzimi sono elencati secondo il numero proposto dalla Enzyme Commission (EC).*

Standard horizontal starch gel electrophoresis was performed on leg muscle tissue, which was crushed in distilled water. The enzymes studied and the buffer systems are given in tabs. I and II. The electrophoretic techniques used are the same reported by NASCETTI et al. (1988).

The following loci and alleles designations were adopted: isozymes were numbered in order of decreasing mobility from the most anodal; allozymes were named numerically according to their mobility relative to the commonest allele (= 100) found in a reference population of *R. latastei* from Lago di Alserio (Merone, Como) (RLM) (>100 = faster mobility; <100 = slower mobility).

A total of 20 presumptive enzyme loci were analyzed: *aGpdh*, *Ldh-1*, *Ldh-2*, *Mdh-1*, *Mdh-2*, *Me-1*, *Me-2*, *Idh-1*, *Idh-2*, *6pgd*, *G3pdh*, *Sod*, *Np*, *Got-1*, *Got-2*, *Ck*, *Adk*, *Pgm-2*, *Ada*, and *Mpi*. The genetic relationships among the studied populations were evaluated using NEI's (1972) standard genetic identity (I) and standard genetic distance (*D*). We did not use the NEI's (1978) unbiased genetic distance (*D* modified for small samples) because we did not score a sufficient number of loci. The genetic variation of the populations was estimated using the following parameters: expected mean heterozygosity per locus (H_e, unbiased estimate; NEI, 1978); proportion of polymorphic loci, at the 99% level (*P*). The time of evolutionary divergence between taxa was estimated using NEI's (1975) formula: $t = 5x10^6D$. Estimation of phenetic relationships among OTUs was performed constructing UPGMA phenograms on the basis of the matrix of Nei's genetic distances (SOKAL & SNEATH, 1963).

Results and Discussion

17 out of the 20 enzyme loci analyzed in this survey, i.e. *aGpd*, *Ldh-1*, *Mdh-1*, *Mdh-2*, *Me-1*, *Me-2*, *Idh-1*, *Idh-2*, *6pgd*, *G3pdh*, *Sod*, *Np*, *Got-2*, *Ck*, *Adk*, *Pgm-2* and *Ada*, were found monomorphic for the same allele in all the samples of *Rana latastei*, thus indicating very low levels of genetic variation in this species. The allele frequencies at the other 3 variable loci are reported in tab. III.

The values of NEI's (1972) standard genetic identity and standard genetic distance for each pairwise comparison are shown in tab. IV. Nei's standard genetic distance among *R. latastei* populations ranges from 0 to 0.077. The three Italian populations from Piedmont (RLC), Lombardy (RLM) and Friuli (RLT) as well as the isolated population from Istria (RLO) are genetically very similar to each other (average *D* = 0.002), with almost identical allele frequencies at all loci (see tab. III). On the other

		Rana latastei				
		RLC (5)	RLM (6)	RLR (8)	RLT (9)	RLO (7)
Locus	Allele					
Ldh-2	*100*	1.000	1.000	0.250	0.944	1.000
	110	0.000	0.000	0.750	0.000	0.000
	120	0.000	0.000	0.000	0.056	0.000
Got-1	*100*	1.000	1.000	0.187	1.000	1.000
	115	0.000	0.000	0.813	0.000	0.000
Mpi	*100*	0.500	0.833	0.375	0.778	0.643
	115	0.500	0.167	0.625	0.222	0.357
H_e		0.028	0.015	0.061	0.024	0.024
P		0.05	0.05	0.15	0.10	0.05

Tab. III - Allele frequencies at 3 variable loci in populations of *Rana latastei*. The sample size is given in brackets in the headings. H_e = expected mean heterozygosity: *P* = proportion of polymorphic loci. For sample designations see "Materials and Methods".
- *Frequenze alleliche a 3 loci enzimatici in popolazioni di* Rana latastei. *Il numero di esemplari studiati per campione è riportato tra parentesi.* H_e = *eterozigosi media attesa:* P = *proporzione di loci polimorfici. Per le sigle dei campioni vedere "Materiali e Metodi".*

	RLC	RLM	RLR	RLT	RLO
RLC	——	0.994	0.935	0.996	0.999
RLM	0.006	——	0.926	1.000	0.998
RLR	0.067	0.077	——	0.930	0.932
RLT	0.004	0.000	0.073	——	0.999
RLO	0.001	0.002	0.070	0.001	——

Tab. IV - Values of NEI's (1972) standard genetic identity (above the diagonal) and standard genetic distance (below the diagonal) between populations of *Rana latastei*. For sample designations see "Materials and Methods".
- *Valori di identità (sopra la diagonale) e di distanza genetica di* NEI *(1972) (sotto la diagonale) tra le popolazioni studiate di* Rana latastei. *Per le sigle dei campioni vedere "Materiali e Metodi".*

hand, a relatively high genetic distance was found comparing the Emilia-Romagna sample (RLR) with the other samples from Italy and Istria (average $D = 0.072$), indicating that this population is genetically recognizable from all the others. This seems presumably due to the geographic origin of the Punte Alberete sample, the population from this locality occurring in the extreme south border of the species range (CAPULA, 1980) and being separated by a relatively broad geographic gap (≈ 100 km) from the other Italian populations. Genetic drift phenomena are to be excluded, as the genetic variability found in the sample from Punte Alberete is higher than that observed in the other samples. While the present distribution range of *Rana latastei* suggests geographic isolation for the populations occurring in Istria, reasons for isolation of the Punte Alberete population are not obvious. Although this is known to be the southernmost *Rana latastei* population as well as the unique population at present occurring in the Emilia-Romagna region (CAPULA, 1980), significant geographical barriers separating this from the other Italian populations do not appear to be present. The Punte Alberete population might simply represent a fragment of the past distribution of *R. latastei* in the southern part of the Padano-Veneta Plain.

The low values of genetic distance found comparing Italian and Yugoslavian *R. latastei* populations reveal that they are genetically quite similar, clustering together

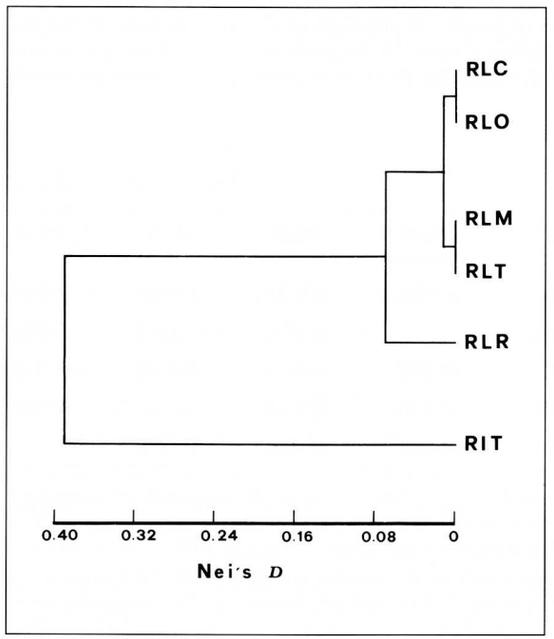

Fig. 2 - Phenogram generated by UPGMA cluster analysis based on NEI's (1972) standard genetic distances between the populations of *Rana latastei*. For sample designations see "Materials and Methods". RIT represents a *Rana italica* sample from central Italy (Tolfa Mountains, Latium).

- *Fenogramma costruito con il metodo UPGMA sulla base delle distanze genetiche di* NEI *(1972) osservate tra le popolazioni di* Rana latastei. *Per le sigle dei campioni vedere "Materiali e Metodi". RIT indica una popolazione di* Rana italica *dell'Italia centrale (Monti della Tolfa, Lazio).*

in the UPGMA phenogram (fig. 2). The degree of genetic differentiation between the studied samples corresponds to that observed comparing populations of the same species, the Nei's *D* values reported in tab. IV being remarkably lower than those found comparing brown frog populations belonging to different species (e.g., average *D* ranges from 0.351 between *Rana dalmatina* and *R. latastei*, to 0.580 between *R. graeca* and *R. latastei*: CAPULA & NASCETTI, unpublished data) (see fig. 2).

According to NEI's formula (1975), the estimated time of divergence between the Istrian and the Italian populations seems to go back to the Upper Pleistocene-Holocene, thus indicating the recent geographic isolation of *R. latastei* in NW Yugoslavia. This strongly supports the hypothesis provided by BURLIN & DOLCE (1986) that the populations occurring in Istria are separated from the Padano-Veneta Plain mainstock in geological recent times.

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