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Adaptations of some amphibian species to Mediterranean environmental conditions

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ABSTRACT. Recent morphological and electrophoretic studies on the amphibian species *Rana ridibunda, Bufo viridis, Hyla arborea* and *Triturus alpestris* from northern and central Greece revealed a high degree of morphological and genetic variation. Morphological variation was assessed by colour patterns and several morphometric characters. Genetic variation was estimated from values of the parameters P (proportion of polymorphic loci) and H (mean heterozygosity), which were found to increase in the order: *Triturus alpestris* (H = 0.122) < *Hyla arborea* (H = 0.136) < *Bufo viridis* (H = 0.139) < *Rana ridibunda* (H = 0.149).

Furthermore, our studies on the reproductive biology of *R. ridibunda, B. viridis* and *H. arborea* revealed some differences concerning their reproductive patterns.

The results of our studies provide evidence that the morphological and genetic variability as well as the differentiation of reproductive patterns are partly adaptations to the ecological factors of the area.

KEY WORDS: Amphibia, reproductive period, morphometrics, genetic variation.

INTRODUCTION

The premises of ecological genetics are that natural populations are adapted to their physical and biological environments, and that the genetic mechanisms respond to environmental change. There are many studies on the phenotypic differences among species of amphibians in different environments, but the genetic factors responsible for these differences are known only in a few cases (DUELLMAN & TRUEB, 1994). The patterns of genetic variation within and among populations of a species may be related either to natural selection, random genetic drift, migration or breeding structure. Many amphibian species are adapted to a variety of environments and these species have been used to investigate the possible relationships between population genetic structure and habitat (AVISE & AQUADRO, 1982; NEVO, 1988; NEVO & BEILES, 1991).

Recent studies carried out on the Greek amphibian species *Rana ridibunda* Pallas 1771, *Bufo viridis* Laurenti, 1768, *Hyla arborea* L., 1758 and *Triturus alpestris* Laurenti, 1768, revealed a high degree of morphological and genetic variation (KYRIAKOPOULOU-SKLAVOUNOU,1980; KYRIAKOPOULOU-SKLAVOUNOU et al., 1991, 1992, 1997; KARAKOUSIS & KYRIAKOPOULOU-SKLAVOUNOU, 1995). This paper is an overview of the above reports, and an attempt is made to relate this morphological and genetic variability to the environmental conditions prevailing in this part of the Mediterranean region .

The reproductive patterns of the amphibians that breed in this region seem to be affected by the environmental conditions. Studies on the reproductive biology of *Rana ridibunda, Rana dalmatina* Bonaparte, 1840 and *Bufo viridis* (KYRIAKOPOULOU-SKLAVOUNOU, 1983; SOFIANIDOU & KYRIAKOPOULOU-SKLAVOUNOU, 1983; KYRIAKOPOULOU-SKLAVOUNOU & KATTOULAS, 1990; KYRIAKOPOULOU-SKLAVOUNOU & LOUMBOURDIS, 1990; KYRIAKOPOULOU-SKLAVOUNOU & ALEXIOU, 1994) showed that these species in Greece exhibit some differences in several components of their reproductive strategies, particularly the length of the breeding period, compared to those reported for the same species in northern Europe (e.g. HEYM, 1974; OBERT, 1975; TESTER, 1990; NOLLERT & NOLLERT, 1992). Besides, it is also known that patterns of reproduction in amphibians are modified by natural selection so as to produce strategies with high fitness, and they reflect a compromise among many selective pressures (DUELLMAN & TRUEB, 1994).

Since almost all the above species are geographically widespread in central and northern Europe, in this paper

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we compare our data concerning the Greek populations with those reported for the northern European ones, in order to point out the differences. In several cases comparisons were also made with conspecific populations from other Mediterranean countries. Some data, particularly those concerning the length of the reproductive periods of *B. viridis* and *H. arborea* are reported for the first time for this region.

MATERIAL AND METHODS

Reproductive period

The data on the reproductive activity and the duration of the breeding seasons of the species *R. ridibunda, H. arborea* and *B. viridis* are based on field observations that were made during the period 1979-1999. Most of our observations have been made in ponds situated close to the city of Thessaloniki, Gallikos river, Chalkidiki (Nikiti & Kassandra) and Lake Kerkini. Occasional observations have been made in many other localities of the country in north and south Greece. All localities situated around the city of Thessaloniki at a distance of 10 to 100 km were visited repeatedly during the breeding season. Our visits were more frequent at the beginning, the end, and the peaks of reproductive activity, and rarely during the remainder of the reproductive period. The temperature in °C of the water in ponds was measured during every visit.

Morphometric and Isozyme analysis

The materials and methods concerning the numbers of populations and specimens, and the morphometric and isozyme analyses have already been described in detail (Kyriakopoulou-Sklavounou et al., 1991, 1992, 1997; Karakousis & Kyriakopoulou-Sklavounou, 1995). All the examined populations are derived from northern and central Greece: three *R. ridibunda* populations from Lake Vistonis, River Gallikos and Lake Ioannina; two *H. arborea* populations from Kerkini and Halkidiki; five *B. viridis* populations from Thessaloniki, Nestos, Halkidiki (Nikiti & Kalandra) and Elassona; four populations *T. alpestris* from Agia Paraskevi, Metsovo, Pertouli and Kapenissi. The collected specimens were transported alive to the laboratory. Morphological variability was estimated by the description of coloration patterns and the measurement of several morphometric characters selected in each case.

Five morphometric characters widely used for discrimination between different taxa in the water frogs were measured in *R. ridibunda:* length of body (L), tibia (T), femur (F), digitus primus (DP), and callus internus (CI). From these characters ten ratios were calculated. Four morphometric characters were measured on the left and right sides of all specimens of *H. arborea:* snout-vent length (SVL), tibia (T) and maximum length (LI) and width (WI) of the inguinal loop. Seven morphometric characters were measured in all specimens of *B. viridis*: lengths of body (L), femur (F), tibia (T), digitus primus (DP) and callus internus (CI), distance between paratoid glands (LPG), and head width (HW). Mean values and standard deviations were computed. Differentiation among populations was investigated using the discriminant analysis (PROCs UNIVARIATE, STEPDISC; SAS 1988). Based on the morphometric characters Mahalanobis distances between the populations were calculated.

Horizontal starch electrophoresis was used for the enzymes and Hb). For the analysis of the serum transferrins and albumins, acrylamide gel electrophoresis was used. Blood samples were taken by cardiac puncture (using EDTA as an anticoagulant), and centrifuged. Skeletal muscle from each frog was homogenized in 0°C phosphate-phenoxyethanol-sucrose buffer using standard procedures (Nakanishi et al. 1969). Homogenates were stored at -25°C. For the electrophoretic analysis, 10% starch gels were used. Three different buffer systems were used. N-(-3-aminopropyl) morpholine/citrate buffer pH 6.1, described by Clayton & Tretiak (1972); for the enzymes, aspartate aminotransferase, malate dehydrogenase and phosphomannose isomerase (AAT E.C. 2.6.1.1, MDH E.C. 1.1.1.37 and PMI E.C. 5.3.1.8). Tris-citrate buffer pH 8,2 described by Ridgway et al. (1970); for the enzymes phosphoglucomutase and lactate dehydrogenase (PGM E.C. 2.7.5.1, LDH E.C. 1.1.1.27, and CK E.C. 2.7.3.2). Tris-LiOH-Boric buffer pH 8.2, described by Ashton & Braden (1961), for the esterases (EST E.C. 3.1.1.-), superoxide dismutase (SOD E.C. 1.15.1.1) and for the haemoglobin (Hb).

For the analysis of the transferrins and albumins, acrylamide gel electrophoresis was used. Conditions were as follows: electrode buffer=0.065 M Tris, 0.031 M Boric acid pH = 8.8; gel buffer=0.35M Tris, 0.056M HCL pH = 8.9. Running gel consisted of 10 ml acrylamide (30% acrylamide- 0.8% bis), 22.5 ml buffer 40 µl TEMED, 20 mg Ammonium persulfate (APS) and stacking gel of 1.5 ml acrylamide 9 ml buffer 10 µl TEMED and 10 mg APS. For the analysis of transferrins, the serum samples were treated overnight with 0.25% Ferrum ammonium sulfate at 4°C.

Electrophoretic data were analysed using the BIOSYS-1 computer package (Swofford & Selander, 1989). Allele nomenclature follows that of Allendorf & Utter (1979).

RESULTS

Reproductive period

The results on the duration of the reproductive periods of the three anuran species studied are shown in Table 1. In this table we present the extreme dates of beginning and end of the reproductive periods that we observed mainly during 1979-1989. Usually, the spawning season

TABLE 1

Maximum length of the reproductive periods of some anuran species in Greece and Central Europe. In parentheses the duration of these periods in months.

Species	**Reproductive period**	
	Greece	**Central Europe**
Bufo viridis	15 February-5July (4.5)	April-June (3)
Hyla arborea	26 February-5 July (4)	April-June (3)
*Rana ridibunda**	23 March-8 July (3.5)	May-June (2)

*The data on *R. ridibunda* are reported in Kyriakopoulou-Sklavounou, P. & M.E. Kattoulas (1990).

of *B. viridis* and *H. arborea* starts at the end of February or the first week of March and lasts until the first week of June, always depending upon the weather conditions. The spawning season of *R. ridibunda* starts about fifteen days later and lasts until the end of June or the beginning of July. It is obvious that all examined species are long breeders. The length of their spawning season ranged from about 3.5 to 4.5 months in northern Greece. In middle European countries their reproductive periods are much shorter (e.g. HEYM, 1974; OBERT, 1975; TESTER, 1990; NOLLERT & NOLLERT, 1992). It should be noticed that in mountain habitats with altitude more than 500 m the reproductive activity of the same species can be delayed from fifteen days to one month.

We observed that temperature is the major factor that affects the initiation and the duration of reproductive activity of the three anuran species. In all cases, when the water temperature suddenly drops below the lowest limit for oviposition, the oviposition is interrupted and starts again when temperature rises. As the weather is unstable in spring, such interruptions may happen many times during the reproductive period. Rainfall, is mostly related with initiation of the breeding activity, particularly of the species *B. viridis* and *H. arborea*. Oviposition occurs when the water temperature ranges from 10°C to 25°C for *B. viridis* and *H. arborea,* and 15°C to 25°C for *R. ridibunda*. The optimal water temperatures for spawning of *B. viridis*, and *H. arborea* ranged between 16°C-20°C and 18°C-22°C, for *R. ridibunda*.

Genetic and morphological variation

Five polymorphic enzyme systems encoding eight loci (AAT-1, AAT-2, LDH-1, LDH-2, MDH-1, MDH-2, PGM, PMI) were found in *R. ridibunda* (KYRIAKOPOULOU-SKLAVOUNOU et al., 1991). From the nine protein systems examined in *H. arborea* that represent 20 gene loci, four were found to be monomorphic (AAT, CK, SOD and Hb) and five polymorphic (ALB, EST, LDH, MDH and TRF) (KYRIAKOPOULOU-SKLAVOUNOU et al., 1992). The six enzyme and three non-enzyme protein systems investigated in *B. viridis* encode 14 gene loci. Five of these loci were found monomorphic in all populations (AAT-1, EST-1, LDH-1, CK-1,Hb-1), and the other nine polymorphic (ALB-1, TRF-1, AAT-2, MDH-1, MDH-2, SOD-1, EST-2, EST-3, LDH-2) (KARAKOUSIS & KYRIAKOPOULOU-SKLAVOUNOU, 1995). Finally, the ten enzyme systems investigated in the alpine newt *T. alpestris*, allowed the scoring of 14 gene loci. Six of them were monomorphic in all examined populations (SOD-1, SOD-2, ME-1, IDH-1, MDH-1, GPD-1) and the remaining eight were polymorphic (AAT-1, EST-1, EST-2, IDH-2, LDH-1, MDH-2, PGI-1, PGM-1) (KYRIAKOPOULOU- SKLAVOUNOU et al., 1997).

The results on the genetic variation of the four amphibian species are shown in Table 2. The mean heterozygosity (H) among the four amphibian species studied ranges from 0.122 to 0.149 and the proportion of polymorphic loci (P) from 37.5% to 50 %. It is evident that the water frog *R. ridibunda* shows the greatest variability for both of the above genetic parameters.

TABLE 2

Genetic diversity of some Greek amphibian species. N: number of specimens (in parentheses the number of populations sampled). H: mean heterozygosity; P: proportion of polymorphic loci.

Species	**N**	**H**	**P**
(Urodela)			
Triturus alpestris	95 (4)	0.122	37.52
(Anura)			
Bufo viridis	112 (5)	0.139	47.12
Hyla arborea	51 (2)	0.136	38.46
Rana ridibunda	110 (3)	0.149	50.00

The results on morphological variation should be separated in two phases: (A) The description of pattern of external morphology and colouration observed in studied populations: (B) The morphometric data and their analysis.

The three populations of *R. ridibunda* differ in colouration, being bright green with a white belly at Ioannina, whereas *R. ridibunda* from Lake Vistonis are pale green with a yellow wash in the groin. The colouration of the Gallikos frogs resembles more that of Ioannina frogs. In morphometric analysis all populations showed a significant degree of morphological differentiation from one another. Of the ten morphometric ratios three, DP/CI, L/T and T/DP were selected in order of precedence as contributing significantly to the discrimination between the populations. The most distinct population was that from Ioannina (KYRIAKOPOULOU- SKLAVOUNOU et al., 1991).

Tree frogs in northern Greece show colour and morphological variability. Colouration varies from bright green to dark brown and many individuals have a green-grey colour with dark spots on their backs. The most striking morphological variation of *H. arborea* in this region is the very narrow inguinal loop that many individuals have;

in some, it is completely absent. This variation is more apparent in populations inhabiting the sea shore zones. Morphometric analysis showed that the two populations differ only in body length, which was larger in the population of Halkidiki; this was the most significant discriminator between populations; when its effect was removed, no other variable differed significantly between populations (KYRIAKOPOULOU-SKLAVOUNOU et al., 1992).

External polymorphism is also great in the toad *B. viridis*. The colour of the dorsal pattern is usually grey or greenish to olive with defined greenish markings, often with dark edges. But in several animals these markings are very pale and in some others they are faint or even absent. Phyletic external polymorphism has also been observed. The females have red spots scattered on all the dorsal surface of their body. External polymorphism in *B. viridis* was greater in the population of Halkidiki, as in *H. arborea*. Discriminant analysis of morphometric characters indicated that the population from Elassona differed significantly from the others. The two variables that contribute most to the differentiation are tibia length (T) and body length (KARAKOUSIS & KYRIAKOPOULOU- SKLAVOUNOU, 1995).

Finally, it should noticed that for the species *R. ridibunda* and *B. viridis* it was found that morphological differentiation is related to altitude. Our studies also showed that morphological and genetic variability are not directly correlated.

DISCUSSION

In Table 1 it is shown that *B. viridis*, *H. arborea* and *R. ridibunda* in this region have very prolonged breeding periods compared to those of central Europe. In particular, it was found that *R. ridibunda* oviposits twice within this prolonged period (KYRIAKOPOULOU-SKLAVOUNOU & LOUMBOURDIS, 1990). According to our observations, temperature seems to be the most important environmental factor that affects the length of the reproductive period of the above species in this Mediterranean region; rainfall is the second one, mainly in relation to the initiation of breeding. Dependence of breeding activity upon temperature was also reported for the species *R. ridibunda* in Germany (e.g. HEYM,1974; OBERT,1975) and for *H. arborea* in Switzerland (TESTER,1990). Consequently, in central and northern European regions where temperature is usually lower, the reproductive period of the above species starts about one month later, and it is shorter. On the contrary, in Italy where climatic conditions are similar to those in Greece, the toad *B. viridis* breeds in March and April (GIAKOMA et al. 1997); *Rana esculenta* L., 1758, a water frog closely related to *R. ridibunda* breeds from late March to early July, very much like *R. ridibunda* in Greece (RASTOGI et al. 1983). From the above, it is concluded that the reproductive period of Greek amphibians is well adapted to the climatic conditions of this region.

Environmental conditions of this region are probably responsible for the genetic and morphological variation that was found in some Greek amphibian species. From the values of the mean heterozygosity (H) it is evident that intraspecific genetic variation increased in the following order: *T. alpestris* (H=0.122) < *H. arborea* (H=0.136) < *Bufo viridis* (H=0.139) < *Rana ridibunda* (H=0.146). These values are much higher than the average values of H reported for Salamandridae (0.058), Hylidae (0.051), Ranidae (0.075) and Bufonidae (0.105) (NEVO & BEILES, 1991). The mean heterozygosity found in Greek populations of *T. alpestris* (H=0.122) is higher than that reported by ARANO et al. (1991) (H_e = 0.109) for *T. a. alpestris* from west and central Europe, and lower than those of KALEZIC & HEDGECOCK (1980) (H_e=0.154) for *T. a. alpestris* in Yugoslavia. High genetic variation was also reported for the same species of anuran in Israel where the average heterozygosity was 0.088, 0.088 and 0.169 respectively for *Rana, Hyla* and *Bufo* (NEVO & BEILES, 1991). According to the above authors, the degree of variation is correlated with the species habitat. Species living under broader and unpredictable ecological spectra, such as *B. viridis* and *H. arborea,* generally have higher values of genetic variation than species living in a more stable environment such as *R. ridibunda.* However, our results do not coincide with those of (NEVO & BEILES, 1991). In Greece, *R. ridibunda* seems to have the highest genetic variation, which was also high in *H. arborea*. Generally, because heterozygosity estimates can be influenced by several evolutionary factors as well as by the choice of loci studied and laboratory conditions, differences in heterozygosity level should be interpreted with caution (AVISE, 1977). However, we will attempt to make some speculations about the factors responsible for this high genetic variation found in our studies. All the examined populations inhabit the same geographical region and face the same environmental factors. Such factors are the climatic conditions and the great heterogeneity of the environment. However, for *R. ridibunda* the average heterozygosity was 0.200 for the Vistonis population, 0.088 for Gallikos, and 0.159 for Ioannina. Thus, other factors should be examined. It is known that at Ioannina lake *R. ridibunda* lives in sympatry and hybridizes with *Rana epeirotica* Schneider et al., 1984 (SCHNEIDER et al. 1984; KYRIAKOPOULOU-SKLAVOUNOU et al., 1991); therefore, the high proportion of polymorphic loci suggests a gene flow from *R. epeirotica* into *R. ridibunda*. Similar reasoning could be adopted for the high heterozygosity at Vistonis lake where probably two species of water frogs, *R. ridibunda* and *Rana balcanica** live also in sympatry. Recently, it was reported that only the lake frogs of Thrace (the most north-eastern part of Greece including Lake Vistonis), belong to *R. ridibunda* while most of Greece is inhabited by a second species named *Rana balcanica* Schneider et al., 1993 (former *R. ridibunda*) (SCHNEIDER et al., 1993; SOFIANIDOU e*t al*., 1994). Another explanation is that the region of Vistonis represents a hybrid zone between *R. ridibunda* and *Rana bedriagae* Camerano, 1882 from Anatolia; it also suggests there is a gene flow from *R. bedriagae* to *R.*

ridibunda. From the above it is evident that more investigation is needed in order to obtain a complete data set.

Finally, it should be noted that the morphological variability revealed in all the examined amphibian species does not correspond to genetic variation. It seems more probable that these variations in colouration patterns in southern European populations could be considered as different ecotypes. The altitude seems to be the major factor that influences the differentiation of morphometric characters in *R. ridibunda* and *B. viridis,* and probably the temperature and aridity in *H. arborea*.

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