

Comparative DNA content in *Discoglossus* (Amphibia, Anura, Discoglossidae)

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With 1 Table

(Accepted: January 27, 1994)

Key words: *Discoglossus*, DNA content, taxonomy

Abstract

The inter- and intraspecific variation of DNA amount of 7 *Discoglossus* taxa was determined by flow cytometric measurements. Comparing the genome size at species level *D. pictus* showed the lowest DNA content, followed by *D. sardus* and *D. montalentii* with similar genomic size. *D. galganoi* has the largest DNA content.

The mean DNA amount of the analyzed specimens ranged from 11.3 pg/cell in *D. pictus auritus* to 15.0 pg/cell in *D. galganoi jeanneae*. DNA content of web tissue in *Discoglossus* was significantly higher than in skin and in the mixed tissue of amputated phalanges. No significant intraspecific differences in genome size between specimens, populations or, respectively, the subspecies *D. p. pictus*/*D. p. auritus* and *D. g. galganoi*/*D. g. jeanneae* were observed. The quantitative differences among the genome of the *Discoglossus* taxa agree with the recent taxonomy.

Introduction

Recently, several taxonomic investigations focused on the mediterranean anuran genus *Discoglossus*. Since 1984 three new species of this genus have been described. LANZA et al. (1986) recognized 8 valid *Discoglossus* taxa:

D. nigriventer: Northern Israel (probably extinct).

D. pictus pictus: Sicily, Malta, and Gozo Island.

D. pictus auritus: Algeria, Tunisia, Southern France/NE Spain (probably introduced).

D. pictus scovazzi: Morocco.

D. sardus: Argentario (Tuscany), Islands of Corsica, Sardinia, Monte Cristo, Giglio and Hyères.

D. montalentii: Corsica.

D. galganoi galganoi: West and Central Spain, Portugal.

D. galganoi jeanneae: Spain south of the Guadalquivir river.

The purpose of this study is to investigate the phylogenetic relationships within the genus *Discoglossus* using data on the quantitative DNA content among and within various *Discoglossus* species. We will describe differences in genome size and

discuss their possible role in evolutionary relationship. Special attention will be directed towards a comparison of this phylogenetic approach with other hypotheses which are based on enzyme electrophoresis, advertisement call analyses and morphometric measurements.

Recent studies on genome size report means and ranges of DNA content for individual species. The values are mostly measured by flow cytometry, which is a rapid and sensitive method for DNA quantification. Cells are stained with a DNA specific fluorochrome and are analyzed for fluorescence, which is directly proportional to the DNA content.

Amphibians exhibit a wide range of variability in nuclear DNA content, even values of closely related species can differ substantially (OLMO and MORESCALCHI 1978). Therefore DNA content can be used as a species-specific character.

DNA content has been studied in three genera of the anuran family Discoglossidae. Values ranged from 10.5 picogram/nucleus (pg/N) in *Discoglossus pictus* from Sicily (OLMO and MORESCALCHI 1978) to 13.4 in *Alytes cisternasii* and 16 in *Alytes obstetricans* (MORESCALCHI and OLMO 1982), 15.6 pg/N in *Bombina variegata scabra* (DE SMET 1981) and 21 in *Bombina variegata* (OLMO and MORESCALCHI 1978).

Relative DNA values were presented by ULLERICH (1970) for *Bombina bombina*.

Material and Methods

Samples of tissue from 15 individuals were available for comparison. The following specimens were studied: 1 male of *Discoglossus p. pictus* (Palermo, Sicily); 1 male and 1 juvenile of *D. p. auritus* (Southern France); 2 males (1 from Mezzana, Corsica, 1 from Sassari, Sardinia) of *D. sardus*; 1 female of *D. spec.* (Sardinia?; see section "The status of *D. spec.*"); 1 male, 1 female, and 1 juvenile of *D. montalentii* (Vizzavona, Corsica); 2 males (1 from the environments of Madrid and 1 from Orense) and 1 female (from La Coruña) of the Iberian *D. g. galganoi*; 1 male and 2 juveniles of *D. g. jeanneae* (near Facinas, Cadiz province).

Tissue samples were cut off from living animals. Since only very small samples were needed, a killing of the specimens was not necessary. Animals survived the procedure without problems, and in some cases several hours later they started calling and mating again. Tissue samples were collected either as small pieces of web or skin or as amputated phalanges.

Methods for tissue preparations, staining and flow cytometric analysis are described in detail by ULLERICH et al. (1988).

Nucleated cells were stained with the DNA specific dye 4',6-diamidino-2-phenylindole (DAPI, Fa. Sigma) in Tris-HCl buffer pH 7.5 containing 0.5% (v/v) Triton X-100. Chicken erythrocytes (CRBC) were used as internal standard. To exclude errors arising from possible variations of dye to DNA molar ratio and changes in sample, flow characteristic reference cells were analyzed simultaneously with the cells under study. Nuclear fluorescence was measured on a PAS II flow cytometer (Fa. Partec, Münster) equipped with an UG1 excitation filter, TK 420 dichroic mirror and a GG 435 barrier filter. Instrumental gain and the voltage of the photomultiplier was adjusted so that the G1 peak of the CRBC was at channel 30. A simple ratio was calculated based on the channel numbers of the G1-peak for the *Discoglossus* samples and the CRBC standard. For the quantitative evaluation of the histogram a computer program (Fa. Partec) was used to calculate the median and the coefficient of variation (CV) of each peak.

It has to be kept in mind that DAPI binds preferentially AT-rich DNA regions and therefore is not suited to calculate absolute DNA values of cells with an AT/GC proportion differing from

50%. However, for the purpose of phylogenetic interpretations a comparison is possible since also a difference in the proportion of AT can be considered as species-specific.

Statistical analysis was performed with the statistic packages SPSS for Windows and Systat for Windows. Differences in DNA content of the three analyzed tissue classes (toe, skin, and web) were tested by analyses of variance (ANOVAs) which are analogous to a t-test for multiple samples. For taxonomic comparison we divided our samples into 5 classes which correspond to the different species *D. pictus*, *D. sardus*, *D. montalentii*, *D. galganoi* and to the doubtful form *D. spec.* The small number of samples made it difficult to test for normality. Therefore in this case we preferred a Kruskal-Wallis single-factor analysis of variance by ranks. The statistical instrument ANOVA allowed us to test the correlation of taxonomic classification of the specimens with their DNA content despite the very small number of available samples.

Results and Discussion

The sex and the absolute DNA content of the seven *Discoglossus* taxa are reported in Table 1. The average of DNA content of all taxa was 13.3 pg/cell and ranged from 11.3 in *D. p. auritus* to 15.0 pg/cell in *D. g. jeanneae* (mean values for each specimen). Divergence of values within the species were 1.9 pg/cell in *D. pictus*, 1.3 pg/cell in *D. galganoi* and 1.2 pg/cell in *D. sardus*. *D. sardus* and *D. montalentii* show similar values, but the ranges of *sardus/montalentii*, *pictus* and *galganoi* do not overlap.

Are these differences species-specific? The fish genus *Xiphophorus* and the salmonid fishes are examples which demonstrate that DNA content variability between individuals of one taxon, between various populations of one species and even between related species can be very small (TIERSCH et al. 1989, JOHNSON et al. 1987). This example would suggest that even comparing single specimens of different taxa could prove species-specific differences. In fact studies on DNA content in amphibians assumed low individual variability. Generally only single specimens of a species were used for genome size determination (eg. BACHMANN and BLOMMERS-SCHLÖSSER 1975).

On the other hand, it has been demonstrated for fish of the genus *Coregonus* that within certain species there can be a large variation between individuals (up to 75% of the mean genome size; range of individual means from 4.4 pg to 10.5 pg, LOCKWOOD et al. 1991).

Despite the small number of examined *Discoglossus* specimens, statistical analysis support the assumption that the observed differences refer to species-specific DNA contents. A Kruskal-Wallis one-way ANOVA of the mean DNA content values of the examined specimens yielded a significance at the 0.01 level. Same was the case when comparing values of all samples by Kruskal-Wallis-ANOVA. Moreover, literature data of a DNA content of 10.5 pg for *D. pictus* (OLMO and MORESCALCHI 1978) fall within the range of our measurements for this species. Therefore taxonomic interpretation of our data seems tenable. However it should be stated that differences in DNA content indicate taxonomic distinctness, while similarities do not necessarily reflect close relationships. It also has to be stressed that the statistical analysis only shows a general significant correlation of the nuclear DNA content of the analyzed specimens with their taxonomic classification; it does not prove the significance of each of the observed differences.

Table 1. Comparison of DNA content of 15 specimens belonging to seven taxa of *Discoglossus*. M = male; F = female; J = juvenile

Taxon	Sex	n of samples	Mean DNA content (pg/cell) (Min–Max)
<i>D. p. pictus</i>	M	4	11.8 (11.5–12.2)
<i>D. p. auritus</i>	M	5	11.3 (10.3–12.2)
<i>D. p. auritus</i>	J	1	11.7
<i>D. sardus</i> (Corsica)	M	3	12.8 (12.4–13.1)
<i>D. sardus</i> (Sardinia)	M	2	13.3 (13–13.6)
<i>D. spec.</i> (Sardinia?)	F	2	14.8 (14.4–15.2)
<i>D. montalentii</i>	M	4	13.5 (13–13.9)
<i>D. montalentii</i>	F	1	12.8
<i>D. montalentii</i>	J	1	13.3
<i>D. g. galganoi</i> (Madrid)	M	4	14.3 (14–14.5)
<i>D. g. galganoi</i> (Coruña)	F	2	13.9
<i>D. g. galganoi</i> (Orense)	M	2	14.6 (14.4–14.9)
<i>D. g. jeanneae</i>	M	2	14.9 (14.6–15.2)
<i>D. g. jeanneae</i>	J	1	14.8
<i>D. g. jeanneae</i>	J	1	15.0

1. Taxonomy

With exception of *Discoglossus pictus scovazzi* and the probably extinct *D. nigriventer* we investigated the DNA content of all valid *Discoglossus* taxa.

Values of Nei's genetic distance (NEI 1972) as calculated by enzyme electrophoresis results (LANZA et al. 1984, CAPULA et al. 1985, LANZA et al. 1986), advertisement call analyses (GLAW and VENCES 1991, VENCES and GLAW in prep.), and data of morphometric variation (CAPULA and CORTI 1993) are available for all forms except *D. nigriventer*. In the following sub-sections we will compare our results on DNA content with these data on several levels. We will investigate relationships of *Discoglossus* at the level of populations, of subspecies and species. After that we will continue by discussing some controversial taxonomic questions.

Level of Populations

Between populations of the same taxon, Nei's genetic distance ranged from 0 to 0.02 in *D. sardus* and *D. p. pictus* (LANZA et al. 1984). Multivariate investigation for morphometric differences between *Discoglossus* populations of the same taxon were not performed up to now. Call differences between populations of *D. sardus* (same material as examined in this paper) can exist, but in the most important parameter, call duration, the differences are not significant (GLAW and VENCES 1991). Same regards populations of *D. g. galganoi*.

Also the DNA content between populations of *D. sardus* showed no differences. Same was the case between populations of *D. g. galganoi*.

Level of Subspecies

Genetic distance between the three subspecies of *D. pictus* ranges from 0.053 to 0.175 (LANZA et al. 1984). No significant call differences were found between these subspecies (GLAW and VENCES 1991). Genetic distance between *D. g. galganoi* and *D. g. jeanneae* was calculated as 0.07. Call differences between *jeanneae* and *galganoi* from northern Spain were relatively small; they were even smaller than those between *galganoi* populations from northern Spain and Madrid (VENCES and GLAW in prep.). However, multivariate analysis of morphometric measurements evidenced differences between the *pictus* and *galganoi* subspecies (CAPULA and CORTI 1993).

The similar DNA content of *D. p. pictus* and *D. p. auritus* as well as the similar values of *D. g. galganoi* and *D. g. jeanneae* give no evidence of specific distinctness of these taxa.

Level of Species

Between *D. pictus*, *D. sardus* and *D. galganoi*, genetic distances from 0.371 to 0.755 were calculated (LANZA et al. 1986). Comparing the distances, *sardus* appears most closely related to *pictus*, and most distant to *galganoi*. *D. pictus* appears in-between *sardus* and *galganoi*, with comparable distances to both forms. Multivariate analysis of morphometric characters resulted in significant variation between *Discoglossus* species (CAPULA and CORTI 1993). Call analyses show clear differences between the three taxa for different parameters. The most important feature, call duration, is lowest in *D. pictus*, higher in *D. sardus*, and highest in *D. galganoi*, with significant differences between the taxa (GLAW and VENCES 1991). This succession *pictus-sardus-galganoi* corresponds well with the DNA content, which is also lowest in *D. pictus*, higher in *D. sardus* and highest in *D. galganoi*.

The relationships of *D. montalentii*

D. montalentii shows very high genetic distances to all the other taxa (0.845–1.074, LANZA et al. 1986). It is also the only form with distinct osteological differentiation (CLARKE and LANZA 1990), and its call is the most divergent (GLAW and VENCES 1991). Some morphometric characters, as enlarged fingertips and longer hindlimbs, are also characteristic for *D. montalentii*. These data seem to characterize the species as a descendant of a different ancestral stock than the *pictus-sardus-galganoi* group (LANZA et al. 1986).

But this hypothesis is somewhat doubtful. By the interpretation of osteological analyses, *montalentii* is also regarded as the most primitive living *Discoglossus*; but *sardus* is grouped as a sister species or a slightly more derived form together with *montalentii* and apart from the "highly derived *galganoi-pictus* sister pair" (CLARKE and LANZA 1990). This conclusion is corroborated by morphometric analysis; *sardus* and *montalentii* share the following characters which place them apart from *pictus* and *galganoi*: longer nostrils, bigger eyes and a broad interorbital distance (CAPULA and CORTI 1993). Regarding colouration, *sardus* and *montalentii* never have a light median stripe on the back, which occurs in populations of *pictus* and *galganoi*.

The morphological and acoustic differentiation of *montalentii* could also be explained by the fact that Corsica is the only place where two *Discoglossus* species (*sardus* and *montalentii*) occur in sympatry and even syntopy. Therefore, acoustic partitioning to prevent interbreeding could be the reason for the call differences (GLAW and VENCES 1991), and adaptation to different habitat types (pools in rocks along mountain streams versus lowland water bodies) could be the reason for the morphological differences between both taxa.

The data on DNA content provide only slight help to solve this ambiguous situation. DNA content of *montalentii* is very similar to that of *sardus*, what could be seen as a support for CLARKE and LANZA'S (1990) hypothesis of a sister group *sardus-montalentii*. However, when using this argument for *pictus* and *galganoi*, we see that they can not be grouped together as suggested by CLARKE and LANZA (1990), since the DNA content values for these two taxa are very different.

The status of the taxon *jeanneae*

In 1986 BUSACK described the taxon *jeanneae* as new species. In his evolutionary scenario, *jeanneae* is more closely related to *D. pictus* than to *D. galganoi*. BUSACK'S *pictus* specimens were collected in Morocco and belong therefore to *D. p. scovazzi*, which in fact we did not investigate in what regards the DNA content. Nevertheless, as DNA amount differences between the remaining two *pictus* subspecies (*pictus pictus* and *pictus auritus*) and *D. g. jeanneae* are higher than between all other forms, a close affinity of *jeanneae* to *pictus* seems very improbable. In addition, data on genetic differences clearly group *jeanneae* with *galganoi*, and genetic differences between the three *pictus* subspecies are very low (LANZA et al., 1986). One more argument against the relation of *jeanneae* to *scovazzi* is that the calls of *scovazzi* are similar to those of the other *pictus* subspecies, and different from those of *jeanneae* (VENCES and GLAW in prep.).

CAPULA and CORTI (1993) by multivariate analysis of morphometric measurements calculated high morphometric distances between *galganoi* and *jeanneae*. In our opinion, however, these results should not be overvalued; they may be due to

a) a high proportion of relatively young (and small) specimens in the *jeanneae* sample (range snout-vent length 31–51 mm); in fact BUSACK (1986) reports a *jeanneae* specimen of 60 mm. Despite this, own data from northern Spain (VENCES unpublished) seem to confirm a larger size of northern *galganoi* specimens. Snout-vent-length range of 82 adult specimens from La Coruña was 44–70 mm.

b) a high proportion of *galganoi* specimens from northern Iberia in the analyzed sample; only 2 *galganoi* specimens from southern or central Spain (north of the Guadalquivir) were measured by CAPULA and CORTI (1993). Thus the observed differences between *jeanneae* and *galganoi* may not be originated by the existence of two well differentiated taxa but due to a continuous cline through the Iberian Peninsula. Such a cline would correspond with the assumption of CAPULA and CORTI (1993) that the differences of *jeanneae* and *galganoi* may in part be explained by ecological causes.

The status of "*D. spec.*"

Two specimens of this form were supplied with the uncertain locality "Sardinia". Up to now only *D. sardus* is known to occur on this island (LANZA et al. 1986). The calls of the male specimen of *D. spec.*, however, differed substantially from those of *D. sardus* (GLAW and VENCES 1991). The mysterious status of these specimens is corroborated by the data on DNA content of the female, which places the form apart from *D. sardus*. Some hypotheses of the identity of these red-backed animals can be found in VENCES et al. (in press).

2. Differences between tissue types

Our data show that the highest DNA contents of each species could mostly be observed in the web-tissue samples. In most cases DNA content of web was about 0.5–1 pg/N higher than in other samples from the same specimen.

ANOVA of all analyzed samples showed a significant influence of the tissue type (toe, skin, and web) on the DNA content values ($p < 0.05$). This influence appeared even more significant ($p < 0.01$) when toe and skin were grouped together and compared with web.

To demonstrate that the significance was really originated by a higher DNA content of web in comparison to both other tissues, we successively carried out ANOVAs after eliminating all data referring to one of the tissue types. Significant differences ($p < 0.05$) were found between web and toe, and between web and skin, but not ($p > 0.5$) between skin and toe. Thus it can be assumed that DNA content of web tissue in *Discoglossus* is significantly higher than in skin and in the mixed tissue of amputated phalanges. An alternative explanation for these results could be a higher proportion of AT-base pairs in nuclei of web tissue.

3. Sexual differences

D. montalentii and *D. galganoi* show larger DNA values in males than in females (0.5–1 pg, same tissue types). Our data are insufficient to prove the significance of this trend, but if was corroborated by further studies it could be regarded as due to heteromorphism of sex chromosomes. Such heteromorphism has been demonstrated in *Discoglossus*, but the differences observed up to now are not suited to explain the DNA amount variations. The acrocentric chromosome pair 14 of *Discoglossus* (probably *pictus*) is homogeneous in males, with both homologous chromosomes having roughly the same size, and heterogenous in females, with one chromosome longer than the other (MORISCAICHI 1964). These data suggest a mechanism of ZZ (male) and ZW (female, with a W larger than the Z), and would predict a larger DNA amount in females.

Maybe other, undiscovered, heteromorphic chromosome regions are responsible for the observed differences. In erythrocytes of *Xenopus* species, DNA content is 1.4–3.7% lower in males than in corresponding females (ULRICH et al. 1988).

4. Evolutionary trends and life history aspects

DNA amounts in the genus *Discoglossus* are relatively high in comparison with other anurans. This is also true in other discoglossid genera. The value of 21 pg/N in *Bombina variegata* (OLMO and MORESCALCHI 1978) is one of the highest in anurans. Average in anurans was calculated as about 9–10 pg/N (BACHMANN and BLOMMERS-SCHLÖSSER 1975).

OLMO and MORESCALCHI (1975) showed that in Caudates (salamanders and newts) the DNA amounts are generally very high (ranging from 30 to 165 pg/N). They argue that a progressive metabolic decay (oxydative metabolism falling to very low levels) may have been selectively advantageous in the habitats proper to Caudates, and that this phenomenon is associated with the increase in the genome and cell size. In anurans a correlation of genome size and developmental rate was demonstrated by GOIN et al. (1968) and OELDORF et al. (1978).

In the Caudata, paedomorphic (neotenus) species have by far the highest contents (OLMO and MORESCALCHI 1975). SMIRNOV (1990) related the high values of *Bombina* with the paedomorphic characteristics found in this genus.

Within *Discoglossus*, the DNA content is about 30% higher in *D. galganoi* than in *D. pictus*. Research should be conducted to obtain data on a) possible paedomorphic features (retarded and reduced ossification) of *D. galganoi* in comparison to *D. pictus* and b) a possibly lower metabolism and developmental rate of *D. galganoi*.

Conclusions

Our results add a new piece to the constantly increasing mosaic of data on *Discoglossus* systematics. Many approaches to *Discoglossus* taxonomy now have been carried out, and there are only few "missing data sets": Among these are comparative cytogenetics, tadpole comparisons and mitochondrial DNA analyses, which have not yet been published. Probably it will soon be possible to carry out an integrative study which tries to use all data for a phylogenetic tree of the genus *Discoglossus*. In amphibians, recently such an integrative analysis was done on the phylogeny of the newt genus *Triturus* (eg. MACGREGOR et al. 1990): Behavioral data were combined with biochemistry, cytogenetics and records of reproductive interactions.

The mediterranean distribution of *Discoglossus* makes a solid phylogeny of this genus very interesting, since it would allow to test different palaeobiogeographic hypotheses. This has already been done by BUSACK (1986), who tried to compare genetic distances between related Spanish and Moroccan animals to answer the question of past land connections between Iberia and Africa. More extended discussions on this subject can be found in LANZA et al. (1986).

There is one more reason why probably so much attention has been focused on *Discoglossus* systematics: This genus is one example for a group of species which are genetically well differentiated but virtually indistinguishable by morphologic features; in fact, still 30 years ago there was a dispute whether 1 or 2 *Discoglossus* species do exist – today we know at least 5 valid species.

This fact contains also some relevance for other fields of biology. Especially physiologists and neurobiologists have often worked in their experiments with *Discoglossus*. Whether the results of different working groups can be compared may also depend on which species have been under investigation, and to find an answer to this question is not easy. Different labs work with different *Discoglossus* breeding stocks, and often their geographic origin is impossible to determine.

Methods available up to now are not suited to determine easily the taxonomic rank of a certain *Discoglossus* specimen: electrophoretic isozyme analysis normally needs reference specimens for comparison, and for call comparison it is necessary to record calls of highly motivated males.

Species determination by means of DNA content can be carried out without killing specimens, and also juveniles and females can be used. Thus DNA content determination is an interesting new method to determine the taxonomic rank of *Discoglossus* specimens.

Acknowledgements

We are indebted to MARIO GARCIA-PARIS (Madrid) and MASSIMO CAPULA (Roma) who contributed with some specimens and to MARKUS MÜLLER (Köln) who helped in keeping frogs. We also wish to thank ANDREAS KASCHUBA and JÖRN SINDERN (Bonn) for some useful advices for the statistical analysis.

Zusammenfassung

Variation im DNA-Gehalt wurde von 7 *Discoglossus*-Arten und -Unterarten mittels Durchflußzytometrie bestimmt. *D. pictus* zeigte den niedrigsten DNA-Gehalt, gefolgt von *D. sardus* und *D. montalentii*, die eine ähnliche Genomgröße aufweisen. Für *D. galganoi* wurden die höchsten DNA-Werte ermittelt. Der mittlere DNA-Gehalt der untersuchten Exemplare lag zwischen 11,3 pg/Zelle für *Discoglossus pictus auritus* und 15,0 pg/Zelle für *D. galganoi jeanneae*. Der DNA-Gehalt in Schwimmhautgewebe war signifikant höher als in der Haut und in dem Mischgewebe amputierter Phalangen.

Signifikante intraspezifische Unterschiede im DNA-Gehalt zwischen einzelnen Individuen, Populationen oder den Unterarten *D. p. pictus* und *D. p. auritus* sowie *D. g. galganoi* und *D. g. jeanneae* ließen sich nicht feststellen. Die ermittelten DNA-Variationen zwischen den Taxa stimmen mit der gegenwärtigen Taxonomie überein.

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