UNIVERSITY OF NAPLES “FEDERICO II”

Faculty of Mathematical, Physical and Natural Sciences

Degree in Biological Sciences

Department of Zoology

**Experimental thesis
in
Comparative Anatomy**

**Characterisation of the S1 satellite DNA
family in *Rana dalmatina***

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ACADEMIC YEAR 1998-1999

Abstract

In this study, the DNA of *Rana dalmatina* was digested with Asp 718I and the two bands of highly repeated DNA produced were cloned and characterised. The largest fragment (494 bp) corresponded to the entire repetitive unit of the major satellite DNA (RdS1a), while the smaller fragment of 385 bp corresponded to the major fragment of RdS1a produced by digestion. A fragment of 332 bp corresponding to the repetitive unit of satellite S1b (RdS1b) was instead achieved by digestion with Eco RV. RdS1b is highly homologous to the corresponding portion of the repetition of RdS1a and presents the first 36 bp repeated and inverted. This suggested that RdS1b would have been derived from satellite S1a by two distinct and subsequent events. Further, the high sequence homology and length between RdS1a and the S1a of *Rana italica* (RiS1a) confirmed the hypothesis that the satellite S1a is antecedent to S1b and inherited from a common ancestor. Southern blots of *R. dalmatina* genomic DNA digested with Asp 718I produced hybrid bands of fragments of different sizes containing in addition to the satellite S1a, also one or more copies of the S1b satellites. The only sequenced band at the moment corresponded to the repetitive unit of the satellite RdS1a + b (826 bp) deleted of the fragment Asp 718I less than RdS1a (109 bp), while the other double bands should almost certainly correspond to repetitive units of satellites RdS1a + 2b and RdS1a + 3b. Our data suggested different satellite DNA organisation in *R. dalmatina*, including the tandem structure of the repetitive units of the RdS1a or RdS1b. Our data also suggested the existence in *R. dalmatina* of at least four different types of hybrid repeating units in all the populations examined.

Acknowledgments

I would like to express my gratitude to my supervisors, Prof. Chinali and Prof. Picariello, alongside Dr. Feliciello, who were always encouraging, patient and generous with their time. I enjoyed their constructive comments, criticisms and discussions. I am indebted to them for being always ready to help me, to discuss and read the draft versions of my thesis. I would also like to thank the whole group of the laboratory of Prof. Chinali that helped me explaining complex methodologies and calculations, and sometimes cheering me up when some results were not particularly encouraging! A special thank goes to Mimmo Lotti and Francesco Itollo for their help in drafting this thesis and the realisation of the figures. Last but not least, my biggest thank is for my parents for having always supported and believed in me!

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1. Introduction

1.1. European brown frogs and their range

Amphibians were the first vertebrates to have adapted to life on land. They managed to survive in multiple biogeographical regions. The western Palearctic region includes Europe, the Mediterranean basin and Asia Minor. The eastern borders are represented by the Ural Mountains, the Ural River, the western and southern shores of the Caspian Sea and the Iranian Plateau to the Indus River, while the southern border roughly corresponds to the Tropic of Cancer (Bartholomew et al., 1911). The species of the genus *Rana* distributed in this region can be divided into two species groups: the group of *R. esculenta* L., 1758 (green frogs) and that of *R. temporaria* L., 1758 (brown frogs). Green frogs are characterised by green background colour, vomerine teeth included between the choanae (internal nostrils) or just beyond their posterior margin, close-set eyes and in a rather dorsal position, males with external vocal sacks, and costumes mainly aquatic.

Brown frogs, on the other hand, have a reddish-brown background colour, with a dark V-shaped temporal spot on the sides of the head, posterior vomerine teeth to the choanae or far beyond their posterior margin, eyes widely spaced and in a rather lateral position, males with internal vocal sacs (which are not everted during singing) or completely absent, and mainly terrestrial costumes. There are 10 species of the latter group in the western Palearctic region: *R. arvalis* Nilsson, 1842; *R. Camerani* Boulenger, 1886; *R. dalmatina* Bonaparte, 1839; *R. graeca* Boulenger, 1891; *R. holtzi* Werner, 1898; *R. iberica* Boulenger, 1879; *R. italica* Dubois, 1985; *R. latastei* Boulenger, 1879; *R. macrocnemis* Boulenger, 1885; *R. temporaria* L.

1758. The classification of these species is based on morphological characters such as the presence of internal vocal sacs, the length of the legs, the shape and degree of development of the metatarsal tubercles, the shape of the muzzle, and different patterns of spots and striae. Most of these characters are often extremely variable within the same species. Many authors, recognising a geographical diversification of these variations, have deemed it appropriate to divide some species into various subspecies. Among all these species of brown frogs, attention was focused on two species: *R. dalmatina* and *R. italica*, and in particular, *R. dalmatina* was studied by comparing the results obtained with those previously obtained from the other species.

1.2. *Rana italica*

R. italica was considered as a subspecies of *R. graeca* only from 1985 by Dubois (*R. graeca italica*), based on some morphological differences between the Italian and Balkan populations (larger in the latter). Previously, no author had hypothesised the existence of two different species, although the ranges of the two frogs were separated. An electrophoretic study of the gene-enzyme systems conducted on these taxa (Picariello et al., 1990) has highlighted significant genetic differences, such as to propose the elevation to a distinct species (*R. italica*) of the Italian population. *R. italica* has a range that includes the whole Apennine chain. Further, this species lacks vocal sacs, and the distance between the nostrils is greater than that between the eye sockets. It also presents flattened or rounded muzzle, tibiotarsal joint, with limb extended forward, reaching or beyond the apex of the muzzle, has small, oval, soft internal metatarsal tubercle 2/5 to 1/2 of the first toe long, and the distal

phalanges are flared in the shape of a T. *R. italica* is almost indistinguishable from *R. graeca* and can therefore be recognised only based on geographical criteria; morphological and osteological researches were also performed (Picariello et al., 1996). As far as ecology is concerned, *R. italica* seems to be more linked to the water of *R. dalmatina*.

1.3. *Rana dalmatina*

R. dalmatina is present in central, southern and eastern Europe. It extends from the French Pyrenees to northern Germany in the north and the Carpathians and European Turkey in the east. It has also been observed around Istanbul (Basoglu and Özeti, 1973) and the Basque Country (Barbadillo, 1987). Isolated populations are present in Denmark and southern Sweden, while its presence in Sicily is doubtful. The precise definition of the range limits is problematic as long-legged individuals of usually short-legged congeners (especially *R. temporaria*, *R. arvalis* and the group of *R. macrocnemis*) are erroneously ascribed to *R. dalmatina*. *R. dalmatina* lacks vocal sacs, and the distance between the nostrils is greater than that between the eye sockets. It also has a pointed or rounded muzzle, ankle joint, limb extended forward, far beyond the apex of the muzzle, large, oval internal metatarsal tubercle, hard and 1/3 to 1/2 of the first toe long. Other characters reported in the literature are not reliable for identification. No intraspecific variability is reported. This species coexists in most of the range with other species of red frogs. However, compared to the latter, it has more distinctly terrestrial customs, which allow it to be distributed in a greater variety of environments. This dispersion may be the reason why *R. dalmatina* is often quite rare compared to its congeners.

1.4. The satellite DNA

It is now common to classify the DNA of eukaryotic organisms according to the number of copies with which a particular sequence is present in the haploid genome. Single sequences are those present once or in a very low number of copies; moderately repeated sequences constitute an intermediate class that reaches up to about 105 copies with an undefined lower limit; highly repeating DNA is made up of sequences repeated more than 105 times per haploid genome (Baldwin and Macgregor, 1985). Satellite DNA is highly repeating DNA that is composed of relatively short repeating units in tandem (Singer, 1982). It is widespread in both the animal and plant genomes and is located in the constitutive heterochromatin of chromosomes, usually at the level of the centromeres or telomeres. This type of DNA is normally not transcribed, although there are exceptions, such as in the lampbrush chromosomes during the oogenesis of *Triturus cristatus* or *Xenopus laevis* (Baldwin and Macgregor, 1985). This highly repeated DNA provided relevant information on phylogenetic relationships between related species (Brutlag, 1980). Its divergence rate is almost always directly proportional to the phylogenetic distance between the species. It is organised with remarkable uniformity within the species, while it diverges between the species with their taxonomic distance.

Unfortunately, its role remains unknown, although a variety of hypotheses have been advanced to explain its function in genomic structure and evolutionary processes. However, an exception to the above can be found in the repetitions located at the end of the telomeres, whose function is well known in the long-term

maintenance of the ends of the chromosomes (Blackburn, 1991). In Amphibians, satellite DNA has been studied mainly in Caudates (Baldwin and Macgregor, 1985; Cremisi et al., 1988; Batistoni et al., 1991; Vignali et al., 1991). The present work was directed to the characterisation of the S1 satellite DNA family in *R. dalmatina*. These highly repeated units show a very high degree of homology, not only with each other but also with the satellite DNA of *R. italica*. The purpose of this work is to study, through molecular analysis, the structure and organisation of this family of satellite DNA within the *R. dalmatina* species. This work is part of a broader study aimed at defining the taxonomic identity of the various species of Palaearctic brown frogs and the phylogenetic relationships existing between these species.

2. Materials and methods

2.1. Chemical reagents and enzymes used

Ampicillin, dATP, dCTP, dGTP, dTTP, buffers A, B, H, L and M for restriction endonucleases, buffer for ligase and hydroxymethylaminomethane (Tris), were provided by the company Boehringer-Mannheim, Sodiolauryl sulfate (SDS) and phenol were supplied by the company International Biotechnologies Inc. Glucose, agar and tryptone were supplied by the company Difco; yeast extract, SeaKem LE agarose and SeaKem HTG agarose were supplied by FMC BioProducts. IPTG (Isopropyl β -D-Thiogalactopyranoside), X-gal (5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside), BCIP (5-Bromo-4-chloro-3-indolyl Phosphate), NBT (P-Nitrotetrazolium Blue) and Extravidine-FITC (Extravidine labelled with Fluorescein isothiocyanate) were supplied by Sigma. All other ultra-pure chemical reagents were supplied by the companies Carlo Erba and J. T. Baker. The restriction endonucleases and DNA modifying enzymes were provided by the companies Boehringer Mannheim, New England Biolabs, BioRad, Pharmacia LKB Biotechnology and Promega. DNase-free RNase is prepared from commercial RNase A (Boehringer Mannheim) according to the protocol described by Feliciello and Chinali (1993).

2.2. Animals

Eight specimens of *R. dalmatina* were used for this study as shown in Table 1:

Rana dalmatina specimens

Male specimens	Site	Date	Female specimens	Site	Date
1	Avellino	19/05/90	1	Girifalco (CS)	30/06/95
1	Catanzaro	24/04/88	2	Margarition, Epirus (Greece)	29/03/88
1	Monte Morello (FI)	11/01/89	1	Montella (AV)	20/02/98
1	Naples	30/06/95			

2.3. Extraction of DNA from tissues

This method was developed by Prof. G. Chinali from previous methods for the extraction of DNA from frog tissues. An aliquot of the fresh tissue sample is weighed (about 200-400 mg), or an aliquot of the sample is weighed in alcohol as such (about 100-200 mg). The sample is crushed into a 15 mL PP test tube (the snap-cap type with round bottom). For every 100 mg of fresh sample, or for every

50 mg of sample in alcohol, 1 mL of GET5 (5% Glycerol, 10 mM EDTA, 25 mM Tris HCl pH 8.0) is added. The mixture is homogenised with repeated mixer strokes, always keeping the tube on ice. A volume of 50 μ L of 10% SDS per mL of GET5 is used (final concentration approximately 0.5%). Then, 25 μ L of proteinase K (20mg/mL) are added per mL of GET5 used (final concentration approximately 0.5 mg/mL). The mixture is then incubated at 50 °C for at least 3 hours until the sample is completely dissolved (digested). Phenol is added in equal volume and the sample is extracted by shaking it moderately for about 5 minutes on a stirrer. After that, 1/10 volume of Na-Acetate 3M pH 5.2 is added, extracted again on a stirrer for a few minutes and centrifuged for 5 min. at 6000 RPM. The supernatant (aqueous phase) is transferred to a new tube and the extraction of the aqueous phase is repeated several times with a 1: 1 volume of phenol-chloroform, until the absence of protein precipitate in the interphase (usually 2-3 further extractions). To recover the DNA present in the residuals not taken from the phases, the first phenolic phase is extracted with half a volume of 0.3M Na-acetate pH 5.2. After centrifugation, the resulting aqueous phase is recovered and used to extract the 2nd phenolic phase and so on until the last phenolic phase is washed. The resulting washing (recovery) aqueous phase is added to the previously extracted aqueous phase. The DNA is then precipitated with 2 volumes of absolute ethanol at 0 °C for 30 min. and centrifuged at 7000 RPM for 5 min. (or the precipitated DNA strands are recovered with a glass rod). All the supernatant is discarded and the pellet is re-suspended in 1 mL of TE for a few hours, or overnight. The pellet is then transferred to 15 mL Corex. DNase-free RNase is added (final concentration 10 μ g/mL) and left at room temperature for at least half an hour. The DAN is precipitated by adding 1 mL (1 volume) of

88% isopropanol - 0.2 M K-acetate. The DNA is allowed to precipitate for at least 10 min. at room temperature. It is then centrifuged for 5 min. at 7000-9000 RPM. The supernatant is discarded and the DNA is re-suspended in 1 mL of TE, then 20 μ L of 5M K-acetate are added and re-precipitated with 2 mL of ethanol (or reprecipitated with isopropanol). A re-precipitation is performed for 20-30 min. at 0 °C and centrifuged for 5 min. at 7000 RPM. The supernatant is removed and re-centrifuged briefly to pipette the residual supernatant. The alcohol present in the DNA precipitate is eliminated by centrifuging the sample leaving the tubes open for 5 min. at 4000 RPM. Remember not to dry genomic DNA completely because it becomes insoluble. The DNA pellet is then re-suspended in 200-500 μ L of TE for some hours at 4 °C. The DNA pellet is centrifuged at 7000 RPM for 5 min. Finally, the supernatant is transferred into labelled and catalogued Eppendorf tubes.

2.4. Bacterial strain *Escherichia coli* XLI-Blue 2

Escherichia coli XLI-Blue 2 (recA1, endA1, gyrA46, thi, hsdR17, supE44, lac⁻, tetr), F '[proAB⁺, lacI^q, lacZ Δ M15, Tn10 (tetr)]. The main feature of the *E. coli* XLI strain (Stratagene Cloning Systems) is a mutation (lacZ Δ M15) which results in the production of an inactive β -D-galactosidase deleted from the N-terminal sequence, and the mutation of the *lacI* gene (*lacI^q*), which leads to an overproduction of the lac operon repressor. The lack of the α peptide of the β -D-galactosidase allows the α -complementation by the pTZ19R plasmid, which expresses the N-terminal part of the lacZ gene, allowing the formation of a functional β -D-galactosidase that determines the blue coloring colonies bearing the intact (non-recombinant) plasmid on plates supplemented with the chromogenic substrate X-gal (5-Bromo-4-chloro-

3-indolyl- β -D-galactopyranoside) and with the IPTG inducer (Isopropyl- β -D-Thiogalattopiranoside). Among the other phenotypic characteristics it is necessary to remember the resistance to tetracycline (tet^r) and the absence of recombinative repair (*recA1*).

The growth of bacterial clones was carried out in LB culture broth (Luria-Bertani medium) consisting of: tryptone 10g/L, yeast extract 5g/L, NaCl 10g/L pH 7.0-7.4 and ampicillin 0.1g/L. The broth is sometimes enriched by adding 1/100 volume of a sterile 20% (w/v) glucose solution. Alternatively, if cells are to be grown on solid medium, plates of LB can be prepared by adding agar (bacteriological) at a concentration of 15g/L to the culture broth, before autoclaving. Any supplements (Ampicillin, IPTG, X-gal) are added after sterilisation in an autoclave, when the medium is at a temperature of 45-50 °C. These supplements serve to keep bacterial cells under particular selective pressure and are generally antibiotics or inducing molecules and to identify clones bearing recombinant plasmids.

2.5. Plasmid vector pTZ19R

In the next chapter we will deal with the various steps for the construction of the recombinant vector. For the moment we limit ourselves to describing the original plasmid (Fig. 1) used as a starting point for our cloning. It is a vector of 2870 bp, it was constructed by inserting the DNA of the intergenic region of the phage ϕ 1 in pUC19 and the promoter sequence T7 near the MCS of pUC19 (Fig. 1A). pTZ19R contains: (1) the origin of replication (*ori*); (2) the *bla* gene, encoding a β -lactamase which confers resistance to ampicillin; (3) a portion of the *E. coli* *lac* operon containing the binding site for the CAP protein, the *Plac* promoter, the binding site

for the lac repressor and the 5'-terminal portion of the lacZ gene encoding the N-terminal portion of β -D-galactosidase. This fragment allows the blue / white selection of the recombinant plasmids. The lacZ gene contains within it a series of unique restriction sites, the “polylinker” (Fig. 1B); digestion of this fragment with one of the restriction enzymes splits it into two parts and prevents the formation of the α region of the β -galactosidase. Therefore, following the addition of IPTG and X-gal, there is no formation of the blue color, due to the cleavage of X-gal, and the white colonies containing the recombinant plasmid are thus selected. The pTZ18R vector is similar to pTZ19R, with the only difference being the inverted polylinker.

Fig. 1 (A) Struttura schematica del plasmide pTZ19R
(B) Sequenza del "polylinker"

2.6. Preparation of competent cells

After having grown the cells in LB to obtain a concentration of about 5×10^7 cells / ml, the culture is centrifuged (5000xg, 3 minutes at 4 °C) and the cell precipitate is re-suspended in 100 mL of a CaCl_2 solution (100 mM) cold and incubated on ice for 30 minutes. The cell suspension is centrifuged (5000xg, 3 minutes at 4 °C) and the bacterial precipitate re-suspended in 7 mL of a sterile solution containing 50 mM CaCl_2 and 15% glycerol. Competent cells are used immediately or quickly frozen and stored at -80 °C until use (once thawed, cells cannot be refrozen).

2.7. Transformation of competent cells

Competent *E. coli* cells are usually transformed with intact plasmid DNA or obtained by in vitro recombination (ligation). Transformed cells are always a small minority and must usually be selected by resistance to one or more antibiotics acquired with the transforming plasmid.

2.7.1. Method for transformation with intact plasmids

A volume of 0.1-1 μL of plasmid, obtained from miniprep, is added to 10-20 μL of water; the plasmid is then incubated with 30-40 μL of competent cells and the mixture is left on ice for 30-40 minutes. Thereafter a thermal shock is caused (2 minutes at 37 °C) and incubated on ice again for 5 minutes. If the antibiotic used for the selection inhibits the synthesis of RNA or proteins, 200-300 μL of LB are added to the mixture thus obtained and it is allowed to incubate for 45-60 minutes at 37 °C. We have observed that incubation is not necessary in case the selection is based on inhibitors of cell wall synthesis, such as ampicillin. The cells are plated at

various dilutions on medium containing antibiotic and incubated for about 18 hours at 37 °C.

2.7.2. Method for the transformation with plasmids derived from ligations

The ligation mixture is precipitated with 1.4 volumes of isopropanol 88% - potassium acetate 0.2M and the DNA is collected by centrifugation (5 minutes at 14000 rpm), discarding the supernatant; the precipitate is then dried by centrifuging the open tubes for 3-5 minutes at 4000 rpm. The DNA is re-suspended in 20 µL of water and used (50-100%) for the transformation. Alternatively, an aliquot (50-100%) of the ligation mixture can be used directly for transformation. The subsequent transformation steps are the same as those seen in the method with intact plasmids, except for the quantity of competent cells that incubate with the plasmid which is equal to 40-80 µL.

2.8. Extraction of microscale plasmid DNA (miniprep)

This method was developed by I. Feliciello and G. Chinali (1993) and is derived from the classic method of alkaline lysis by H.C. Birnboim and J. Doly (1979) with various modifications, which allow to obtain plasmid DNA in a simple and rapid way with a higher yield and in a highly purified form (such as to be practically equivalent to that obtained with the isopycnic gradient of CsCl 6M).

1A) The clones are grown at 30-37 °C O.N. in 10 mL of LB-antibiotic in 50 mL Falcon tubes, the cells are centrifuged for 5 minutes at 4000 rpm, re-suspended in 1 mL of GET5 (Glycerol 5%, EDTA 10 mM, Tris-HCl 25mM, pH 8.0) cold and transfer into 2 mL Eppendorf tubes (liquid cultures).

1B) The clones are grown at 30-37 °C for 16-20 hours on LB-antibiotic plates (4 clones per plate in patches of about 10 cm²), the cells are removed with buffer and re-suspended in 1 mL of GET5 cold in 2 mL Eppendorf tubes (plate cultures).

2) The cells are centrifuged for 1 minute at 8000-10000 RPM at 4 °C, the supernatant is aspirated away and carefully re-suspended in 500 µL of GET5 at 0 °C(3-4 times on vortex keeping on ice in the intervals).

3) Add 250 µL of 0.4M-SDS 2% NaOH, close the tube, inverting it twice to mix the contents, and immediately put it back on ice for 5-10 minutes.

4) Add 750 µL of a cold solution of 4M potassium acetate-2M cold acetic acid, shake the tubes immediately and vigorously by hand 4-5 times, put them back on ice and leave there for about 10 minutes.

5) At this point the samples are centrifuged in a pre-cooled centrifuge at 0-4 °C for 5 minutes at 13000-15000 rpm, 1.4 mL of supernatant are transferred into a new tube, taking care to avoid any floating particles of precipitate.

6) From this step on, you can operate at room temperature. 700 µL of isopropanol are then added, mixed and centrifuged for 5 minutes (1 minute at 5000 rpm, then 4 minutes at 13000 rpm); the supernatant is poured out, the tubes are re-centrifuged in the same orientation for 30 seconds at 13000 RPM and the residual supernatant is aspirated away.

7) The precipitate 250 μ L of TE (Tris 10 mM pH 8.0; EDTA 1mM, pH 8.0) containing 10 μ g/mL of RNase DNase-free is re-suspended and left for 15 minutes at room temperature.

8) The whole is precipitated with the addition of 300 μ L of isopropanol 88% - potassium acetate 0.2 M and left for 10-15 minutes at room temperature.

9) The centrifuge is centrifuged for 5 minutes at 13000 rpm, the supernatant is quickly poured off, the tubes are immediately put back in the centrifuge with the same orientation, re-centrifuge for 1 minute always at 13000 RPM and the residual supernatant is eliminated.

10) To dry the precipitate, the opened tubes are put back into the rotor without lid, centrifuged for 3-5 minutes at 3000-4000 RPM and resuspend the samples each in 50-100 μ L of TE.

2.9. Digestion of DNA with restriction endonuclease

Restriction endonucleases (the ones we use are supplied by Boehringer Mannheim) are enzymes capable of hydrolysing the internal phosphodiester bridges of DNA, recognising particular sequences and cutting the DNA within them or not far away. The functioning and activity of the different restriction endonucleases vary according to the buffer used for incubation; in fact, various buffers are used, which differ in composition, different ionic strength and pH. A restriction endonuclease unit is defined as the amount of enzyme that completely digests 1 μ g of DNA of λ phage in 1 hour at 37 °C. However, this activity is verified on ultrapure DNA preparations with a defined number of sites for the various enzymes. It is

therefore generally useful to use a quantity of enzyme 2-5 times higher than the normal value (2-5 U/ μ g of DNA). The incubation time we use to digest DNA varies from 1 to 16 hours. If digestion needs to be stopped (in the case, for example, of partial digestion), this can be done by adding EDTA in excess of the Mg^{2+} ions present in the solution.

2.10. Agarose gel electrophoresis

Agarose gel electrophoresis is a fast and effective method for both determining the relative molecular weight of DNA fragments and for separating DNA fragments of different sizes. The migration speed of the fragments depends on the following factors: molecular weight, concentration of the agarose gel, conformation of the DNA, conductivity of the medium and intensity of the applied electric field. The concentration of the agarose gels we use generally oscillates between 1 and 2.5% and the formation of the gel, as well as the electrophoretic run, are carried out in TAE buffer in the presence of traces of ethidium bromide ($\sim 1\text{mg/L}$). At the end of the electrophoresis the gel is observed under the UV light transilluminator and possibly photographed.

2.11. Purification of DNA fragments

After suitable digestion, the DNA is subjected to preparative electrophoresis, the useful bands are cut from the gel and the DNA of interest is recovered using GeneClean II.

2.11.1. Purification of linearised vectors

1A) The vector is digested with one or more restriction enzymes.

1B) If one of the sites is to be filled in, this site is digested first, the DNA is precipitated with 1.2-1.4 volumes of isopropanol 88% -potassium acetate 0.2M, centrifuged at 13000 rpm, the tubes are dried, the "fill-in" reaction is carried out with Klenow DNA polymerase (1-2 units/tube) and with the 4 dNTP 0.1-0.5mM for 30 minutes at room temperature, it extracts with phenol-chloroform, precipitates with absolute ethanol, washed with 70% ethanol, dried and then digested with the second enzyme.

2A) The digestion is stopped with 1/4 volume of Dye 5X, the sample is loaded onto a 1% TAE Sea Kem agarose preparatory gel and is subjected to electrophoresis for 60-90 minutes at 130-150 V keeping the light-protected gel.

2B) If the volume of the sample is too large, it is precipitated with absolute ethanol, it is recovered by centrifugation (5 minutes at 4 °C and 13000 RPM), the precipitate is dried well, it is re-suspended in 30-40 µL of TE and then proceed as above.

3) After electrophoresis, the portion of the gel containing the fragment is cut off and placed in Eppendorf tubes and weighed.

4) Add about 400 µL of KI 3M for every 100 mg of gel, incubate at 45-50 °C for a few minutes until all the gel has dissolved, add 5-10 µL of resin (glass -milk) after having well re-suspended it on vortex. The suspension is mixed on a shaker for about 10 minutes (adsorption of the DNA to the resin).

5) The tubes are centrifuged for 10 seconds at 13000 RPM, all the supernatant is removed and the resin is re-suspended on vortex with 400 µL of NEW Buffer (a

solution of salts in 95% ethanol with a pH of 7.0- 8.5, which is stored at -20 °C), re-centrifuge for 10 seconds at 13000 RPM and the supernatant is discarded.

6) The washing with NEW Buffer is repeated two more times; after having discarded the last supernatant, centrifugation is repeated and the last traces of NEW Buffer are completely eliminated (this last step is important to eliminate the salts of the buffer).

7) Re-suspend the resin in 10 µL of TE, held for 2-3 minutes at approximately 45 °C, centrifuge for 20 seconds at 13000 RPM and transfer 10 µL of TE with the DNA into an Eppendorf tube; the extraction is repeated with 10 µL of water and the two supernatants are combined.

8) To eliminate any traces of resin, re-centrifuge the 20 µL of DNA solution for 2 minutes at 13000 RPM, quickly collect all the supernatant and transfer it to a new tube.

2.11.2. Purification of small fragments

Proceed as for large fragments except that electrophoresis is performed on 1.5-2.0% gel. Usually 2-5 µL of resin (glass-milk) are used.

2.12. Ligation of DNA fragments with "blunt" or "cohesive" ends

Ligation is a reaction that leads to the formation of phosphodiester bridges between the 3'-OH end and the adjacent 5'-P end in DNA. The enzyme usually used to catalyse this reaction is T4 DNA ligase (Pheiffer and Zimmerman, 1983). The reaction is usually used to insert foreign DNA fragments into vectors, plasmid or viral. For the ligation of DNA fragments, samples are used, digested with the

appropriate enzyme, precipitated with ethanol or isopropanol and re-suspended in water or TE or purified by the preparative gel. In some cases, it may be convenient, after digestion of the vector, to proceed with a dephosphorylation of its ends to prevent them from binding, thus reforming a vector without an insert. The reaction mixture contains, in 20-40 μL of final volume, 0.2-2 μg of DNA and 1-4 units of T4 DNA ligase. The buffer used (T4 ligase buffer 1X at pH 7.6) consists of: Tris-HCl 66mM, MgCl_2 5mM, DTT (dithiothreitol) 1 mM, ATP 1 mM. The mixture is incubated at 14 °C for approximately 16 hours. Aliquots of this mixture can be used for transformation as such or after precipitation with isopropanol and resuspension of the DNA in the desired volume of water. The differences between the ligation of blunt-ended fragments and that of cohesive-ended fragments mainly concern the different efficiency of the reaction (greater for the latter). For this reason, the quantities of ligase and DNA used are generally 3-10 times greater for blunt-ended fragments.

2.13. Precipitation of DNA with alcohols

DNA can be precipitated with ethanol or isopropanol. The precipitation of nucleic acids requires NaCl salts, sodium acetate, potassium acetate, etc.), which are generally used in concentrations between 0.1 and 1M. The percentage of alcohol required tends to decrease as the concentration of the salt and the size of the polynucleotides increase. The precipitate in ethanol has greater volume and is more soluble in water than the precipitate in isopropanol. Isopropanol precipitates small fragments of DNA less well.

2.13.1. Precipitation with ethanol

It is used to quantitatively recover DNA, even small ones, according to the following procedure: 1) 1/20 of a volume of 5M potassium (or sodium) acetate or 1/10 of a volume of 5M NaCl is added to the DNA solution.

2) 2.5 volumes of cold ethanol are added to the solution obtained, mix well and kept at -20 °C for 1-16 hours (the more diluted the solutions, the longer the precipitation times).

3) It is centrifuged at 10000-14000 RPM for 5-10 minutes at 4 °C and the supernatant is discarded.

4) If the DNA is to be used with enzymes, the precipitate is washed with 70% ethanol, centrifuged at 10000-14000 RPM for one minute at 4 °C, discarded the supernatant and re-suspended in TE after briefly drying the precipitate.

2.13.2. Precipitation with isopropanol

This precipitation is recommended when it is desired to eliminate nucleotides and oligonucleotides in DNA. It is not recommended for recovering very small DNA fragments. This precipitation is usually done at 0-25 °C on the counter or in the fridge, but not in the freezer with the following procedure: 1A) If the DNA is in a solution containing 1-2 M of salt, 1/2 volume of isopropanol is added.

1B) If the DNA is in solution without salts, add 1.0-1.5 volumes of isopropanol 88% -potassium acetate 0.2 M.

- 2) The solutions are mixed well and allowed to precipitate at 0-25 °C for a time ranging from 15 minutes to 2 hours (longer times and lower temperatures are used for very diluted solutions).
- 3) It is centrifuged at 10000-14000 RPM for 5-10 minutes at room temperature and the supernatant is quickly discarded, taking care not to lose the precipitate, which is not very visible.
- 4) If the DNA comes from a precipitation with a low salt concentration (point 1B), go directly to point 5; if the DNA comes from a precipitation with a high salt concentration (point 1A) and must be used with enzymes, the precipitate is washed with cold 70% isopropanol, centrifuged for 5 minutes at 10000-14000 RPM and the supernatant is discarded.
- 5) The precipitate is re-suspended in TE after briefly drying it (centrifugation at lowspeed with the tubes open).

2.14. Transfer and hybridisation of DNA on filter (Southern Blot)

This method allows, through transfer and hybridisation of the DNA on a filter, to be able to identify fragments (monomers and possibly multimers of them) that otherwise would be little or not at all visible in the gel electrophoretic separations. The technique we use is the classic one described by Sambrook et al. (1989).

Electrophoresis

- 1) The genomic DNA sample (usually digested ON with 2-3 U of the restriction enzyme / μg of DNA) is run on an agarose gel in 1.5% TAE (with ethidium

bromide) to 50-100 volts up to the desired distance by placing a standard of known fragments in at least one of the two external wells.

2) At the end of the run, the lower right corner of the gel is cut, then the gel is photographed with a transparent ruler placed near the standard as a migration reference for the blot.

Transfer to filter

1) DNA is denatured by leaving the gel in 200 mL of denaturing solution (0.4M NaOH-1.5M NaCl: 40 mL NaOH 2M + 60 mL NaCl 5M + 120 mL H₂O) for 30 min. with gentle agitation (on rotating plate).

2) The denaturing solution is discarded, the gel is rinsed briefly with bidistilled water and then it is neutralized for 30 min. with gentle stirring in 200 mL of 1M Tris-HCl, pH 7.4-NaCl 1M (100 mL of 2M Tris pH 7.4 + 40 mL 5M NaCl + 60 mL H₂O).

NOTE: step 2 can be skipped if Nylon membranes are used.

3) Using gloves, cut a piece of nitrocellulose or nylon filter 1-2 mm wider than the gel (or portion of gel) to be transferred. The filter is moistened by first placing it on the surface of double distilled water, then leaving it for 5 min. in transfer solution.

4) Prepare about one liter of the transfer solution (usually 10X SSC; 20X SSC is used to transfer fragments of less than 0.5 Kb onto nitrocellulose). The 3MM paper bridge is prepared and, once it is completely soaked in the solution by capillarity, any air bubbles are eliminated with a pipette or glass rod. The level of the transfer

solution in the side trays should be as high as possible (1-2 cm below the 3MM paper plane). The transfer solution can be recovered and reused.

5) Place the inverted gel (face down) on the 3MM paper. The outer edges of the gel are delimited with strips of Parafilm, then lean over the nitrocellulose or Nylon filter and cut (if not done before) the corner of the filter corresponding to the cut one of the gel (to indicate the orientation of the gel). Two 3MM sheets wetted in 2X SSC of the same size as the gel are placed on top of the filter. A layer of 5-6 cm of dry absorbent paper cut to the size of the gel is placed over everything and, finally, a glass plate with a weight of about 0.5 kg on top. The transfer is made for at least 8 hours (preferably ON).

6) The nitrocellulose or Nylon filter containing the DNA transferred from the gel is peeled off and left for 5 min in 6X SSC with gentle agitation to remove any gel residues. The washing with fresh 6X SSC is repeated if the alkaline transfer with a Nylon filter has been performed (see note above). The filter is dried on absorbent paper for a few minutes, then placed between two 3MM sheets and “cooked” for 1-2 hours at 80 °C under vacuum (fixing of the DNA to the filter). The filter can be stored dry and under vacuum until use (in the glass bell).

Probe preparation

- 1) The probe labelled with biotin is prepared.
- 2) The quantity of the probe is determined.

3) The probe aliquot required for hybridization is taken (usually 5-10 µg for biotinylated probes) and denature it before use.

4) Denaturation in H₂O can be done for 5 min at 95 °C, usually in the presence of 5-10 µg of non-homologous carrier DNA (from salmon sperm or *E.coli*) or for 2-5 min at 100 °C in 0.4 M (final) NaOH. After denaturation (which must be performed immediately before use) the probe is immediately placed on ice and kept at 0 °C before adding it to the hybridization mix.

Pre-hybridisation and hybridisation

1) The filter is left to rehydrate by floating it on 6X SSC and then leaving it immersed for a few minutes.

2) The filter is pre-hybridised under gentle shaking for 1 hour at 68 °C with 0.2 mL/cm² (about 15 mL) of pre-hybridisation mixture (6X SSC + blocking reagent 1% + N-lauroylsarcosine 0.1% + SDS 0.02% + fragmented and denatured DNA of salmon sperm 20µg/mL) in a sealed bag. If pre-incubation is performed in a "rolling bottle", care must be taken to put the face of the DNA transfer filter (the one in contact with the gel) inwards. At the end, the pre-hybridisation mix is discarded if Nylon membranes are used.

3) Hybridisation is performed by incubating the O.N. at 68 °C under gentle stirring with the hybridisation mix. This mix is achieved simply by adding the freshly denatured probe to the prehybridisation mix. If nylon membranes are used, it is

better to discard the pre-hybridisation mix, replacing it with half volume of the same fresh mix containing the denatured probe.

NOTE: Hybridisation can also be performed at 42 °C using 45-50% formamide. Hybridisation efficiency can be increased by using 5% dextran sulfate. For cleaner results Denhardt's solution can be used in place of blocking reagent or dry milk.

Filter washing

1) 500 mL of 0.2X SSC - 0.5% SDS are preheated in the thermostatic bath to bring it to the washing temperature.

2) 500 ml of 2X SSC - 0.1% SDS are prepared. After each wash, all the solution is removed and replaced with new fresh wash solution.

3) The hybridisation mix is discarded and the filter is rinsed twice at room temperature with 100 mL of 2X SSC - 0.1% SDS for 5 min in a bowl under gentle agitation.

4A) Very stringent washing: the double washing is repeated as in 3), but in the bathroom at 68 °C, for 15 min. and using the preheated 0.2X SSC - 0.5% SDS solution (see above).

4B) Medium stringent wash: like very stringent, but at 55 °C.

4C) Not very stringent washing: like the medium stringent, but in 2X SSC - 0.1% SDS.

5) The filter is rinsed for 5 min with 100 mL of 2X SSC - 0.1% SDS at room temperature. The detection can be done the next day.

Revelation

Probes labelled with biotin or digoxigenin

- 1) The filter is rinsed briefly (1 min) in 50 mL of Buffer 1
- 2) Incubate for 30 min at room temperature with 50 mL of Buffer 2 (Buffer 1 + 2% dry milk, or 2% albumin or 1% blocking reagent) with gentle agitation.
- 3) The solution is discarded and replaced with a 1 µg/mL solution of streptavidin - alkaline phosphatase conjugated in Buffer 1 (original solution 1 mg/mL). For 100 cm² of filter 7 mL of solution is used. The probes are incubated for 15 min at room temperature with gentle agitation and repeating the solution on the filter from time to time.
- 4) The filter is rinsed 4 times for 15 min. under mild agitation: the first time with 50 mL of Buffer 2, the other three times with Buffer 1.
- 5) Rinse for 5 min. in Buffer 3 to equilibrate the membrane with this buffer.
- 6) A solution in Buffer 3 of NBT 0.33 mg/mL + BCIP 0.17 mg/mL (approximately 7.5 mL/100 cm² of filter) is prepared immediately before use. To prepare it, 0.1 mL of NBT and 0.065 mL of BCIP are added to 10 mL of Buffer 3. The solution is added to the filter and the color is developed in the dark for 0.5 - 4 hours, preferably in a sealed bag (the hybridization bag washed with water can be reused).

NOTE: If a more gradual color development is desired, NBT and BCIP at 1/5 - 1/10 of the indicated concentration can be used.

7) The filter is washed with water to stop the staining reaction. The filter is photographed and/or photocopied before storing it.

Buffer 1: (biotin) 0.1M Tris-HCl pH 7.5, 0.15M NaCl

(digoxigenin) 0.1M ac. maleic-NaOH pH 7.5, 0.15M NaCl

Buffer 2: Buffer 1 + 2% dry milk, or + 2% albumin, or + 1% blo. react.

Buffer 3: 0.1M Tris-HCl pH 9.5, 0.1M NaCl, 0.05M MgCl₂

2.15. Quantitative Dot-Blot

This method allows to evaluate the quantity of a particular DNA sequence in a genome. This technique does not differ substantially from that of Southern Blot, the probe labelled with biotin and the analysis method is the same, the only difference is the probe which is always complemented with non-homologous *E. coli* carrier DNA to always have the same quantity and thus avoid errors due to differences in hybridisation kinetics.

2.16. Non-radioactive in situ hybridisation

This method (also called hybridisation or cytological hybridisation) consists in the denaturation of the DNA of the cells crushed on the slide so that it can react with a single-stranded DNA (or RNA); the added solution is non-radioactively labelled and its hybridisation is followed by means of the M.O. With this type of technique it is possible to directly determine the region of the chromosome in which

a particular sequence is located. The slides were provided by prof. Odierna, obtained from blood cultures of *R. dalmatina* (specimens from Montella).

Pre-treatment of slides

1) They are treated with RNase (100 µg/mL) in 2x SSC pH 7, for 1 hour at 37 °C.
(optional)

2) Washed 3 times for 10 min in 2x SSC.

3) They are dehydrated in 50%, 70%, 95%, and absolute ethanol, for 10 min each step.

Denaturation of chromosomes

1) The slides are treated, dehydrated and preheated, for 3 min. at 80 °C, under coverslips, with 200 µL of a 70% / 2x SSC solution of Formamide, pH 7.

2) They are washed quickly in 2x SSC, pH 7, on ice.

3) The slides are dehydrated by successive steps of 3 min. in ethanol at 50%, 75%, 100%, at 4 °C. They are left absolutely until the moment of hybridisation. Before applying the probe, they dry quickly by evaporation.

Probe denaturation

IMPORTANT: The biotinylated probe is prepared as follows:

Probe 1-3 µL per slide, DNA E. coli 5 µl, Sodium Acetate 3M 1/10 vol., Ethanol abs. (-20 °C) 3 vol.

- 1) It is mixed on vortex, then it is kept for 15 min. at -80 °C.
- 2) It is centrifuged for 15 min. to +4 and the supernatant is discarded.
- 3) It is centrifuged for 2 min. and the residual supernatant is discarded.
- 4) It is centrifuged for another 2 min. with open tubes.
- 5) It dissolves in hybridisation mix (about 30 µL per coverslip, 35 µL per slide).
- 6) The probe is denatured at 100 °C for 7 min, then it is cooled to 0 °C on ice, centrifuged briefly and pipetted onto the slide.

Hybridisation mix (for 10 mL to be aliquoted and stored at -20 °C):

1 mL 20x SSC pH 7, 0.4 mL 1M phosphate buffer pH 6.8, 0.1 mL SDS 10%, 0.1 mL Denhardt, 5 mL deionised formamide, 1.4 mL H₂O, 2 mL dextran sulfate 50%.

IMPORTANT: filter before adding dextran sulfate. Adjust the pH to 7 with phosphoric acid.

***In situ* hybridation**

- 1) Hybridisation is carried out for 20 hours at 42 °C in a saturated atmosphere of 50% Formamide / 2x SSC.

Washing

- 1) The slides are washed twice in 50% Formamide / 2x SSC pH 7, for 2 min. at 42 °C, to eliminate the unbound probe.

2) The slides are washed three times in 2x SSC pH 7, for 3 min. at 42 °C, to eliminate the formamide.

Immunocoloration

1) Blocking: the slides are incubated for 30 min. at 37 °C in a humid chamber with 200 µL of blocking solution: BSA 3% / 4x SSC / 0.1% Tween 20

2) Highlighting: conjugated extravidine-FITC (SIGMA) is diluted to the final concentration of 5 µg/mL (dil 1: 200) in: 4x SSC / 1% BSA / 0.1% Tween 20, pH 7. Yes incubate the slides with extraravidine in 200 µL for 30 min. at 37 °C.

3) Wash the slides in 4x SSC / 0.1% Tween 20, three times for 5 min. at 42 °C, in the dark.

Contrast coloring

1) The slides are immersed (or covered with 0.2 mL) after the third wash in: Propidium Iodide (SIGMA) at the final concentration of 300 ng/mL in 2x SSC pH 7, for 2 min. at room temperature and in the dark.

2) The slides are washed briefly in PBS.

3) They are assembled in Antifade (containing Propidium 200 ng/mL).

Antifade (per 10 mL of stock solution): 0.233 g of DABCO (1,4-diazabicyclo (2.2.2) octane, SIGMA), 200 µL of Tris-HCl 1M, pH 8, 800 µL of H₂O dist., 9 mL of glycerol. Store at 4 °C in the dark.

3. Results

3.1. Cloning and molecular characterisation of satellite DNA

We performed a Southern blot for comparison between *R. italica*, previously studied (Cardone et al., 1997), and *R. dalmatina* (Fig. 2). The restriction enzymes used for the blot were five (Asp 718I, Eco RV, Nde I, Nhe I and Stu I), and, more precisely, those that normally presented cleavage sites at the level of the S1 satellite DNA family in Palearctic brown frogs. For this analysis, *R. italica* S1a probes were used which showed a strong hybridisation also with *R. dalmatina* (Cardone et al., 1997). *R. italica* contains satellite DNAs S1a (RiS1a) and S1b (RiS1b) with repetitive units of 494 bp and 281 bp, respectively. The repeating unit of S1a contains two Asp 718I (Kpn I) sites and one site for Eco RV, Nde I and Stu I, while that of S1b contains only one Asp 718I site and one Stu I site. The patterns of hybrid bands on the Southern blot of *R. italica* DNA digested with the above five enzymes are in perfect agreement with the predictions. At the level of the first lane (Asp 718I digestion), there is a very weak band of about 0.1 kb, a more marked one of about 0.28 kb, a third, also very strong, of about 0.38 kb, a fourth of almost 0.5 kb, and a fifth of about 0.56 kb, in addition to other weaker bands of higher molecular weight. These bands correspond to monomer (281 bp) and dimer (562 bp) of the repeating unit of RiS1b, and the monomer of the repeating unit of RiS1a (494 bp) and the minor (109 bp) and major (385 bp) fragment of this repetitive unit. The patterns generated by digestion with Eco RV and Nde I (lanes 2 and 3), are essentially identical to each other and show only the bands corresponding to integer multiples of the repetitive unit RiS1a (494 bp, 988 bp, etc.).

Fig.2 Southern blot di DNA genomico di *R. italica* e *R. dalmatina* digerito con gli enzimi di restrizione Asp I, Eco RV, Nde I, Nhe I, Stu I.
Corsie: 1,6=Asp I; 2,7=Eco RV; 3,8= Nde I;
 4,9=Nhe I; 5,10=Stu I

As expected Nhe I does not cut the S1 satellites of *R. italica* (lane 4). The fifth lane (Stu I digestion) shows both the 281 bp band of RiS1b and the 494 bp band of RiS1a and further bands of higher molecular weight, corresponding to the multimers of the two satellite DNAs. Finally, by examining again the Southern blots of the DNA of *R. italica* digested with Echo RV and Nde I, it is possible to notice a slight band at about 0.78 kb which indicates the presence of a hybrid satellite DNA with repetitive units formed by S1a + S1b (RiS1a + b). Analyses previously performed on various specimens from different locations (Cardone et al., 1997) have shown that the amount of hybrid S1 satellites can vary greatly even among individuals from the same location. It was thus concluded that the satellite DNAs S1a and S1b were mostly organised in separate groups in tandem in *R. italica*, although a small and variable part of these satellites appeared to be organised in repetitive units containing one or more copies of both S1a and of S1b.

After having described the patterns observed in *R. italica*, we went to examine those of *R. dalmatina*. It was immediately observed that there are only three restriction enzymes that make cuts; in fact, in addition to Nhe I, also devoid of sites on the satellite DNA S1 of *R. italica*, also Stu I does not present bands. As regards digestion with Asp 718I (or with its isoschizomer Kpn I), a very weak band can be seen, at about 0.1 kb, and two bands at about 0.38 kb and 0.50 kb which present the same characteristics and dimensions of the bands of RiS1a and its two fragments already seen in the same digestion in *R. italica*. So it should be *R. dalmatina* satellite DNA S1a (RdS1a). On the other hand, we did not observe any signal at the 281 bp band of *R. italica*. In the same lane, a series of doublets of bands can be observed,

indicating the presence of complex repetitive units. Particularly noteworthy are the doublets having dimensions slightly higher than 0.7 and 0.8 kb and slightly lower than 1.1 and 1.2 kb. From the analysis of the blots of the digested DNA with Eco RV and with Nde I, on the other hand, we noticed the presence of a new fragment, having dimensions of about 0.33 kb, an indication of the probable existence of an S1b satellite and the expected presence of the 0.5 kb band, of the putative satellite S1a of *R. dalmatina*. Among the other bands of higher molecular weight, it is possible to observe in both blots, in addition to the multimers of the repetitive units of the putative satellites S1a and S1b, also bands of different sizes that confirm the existence of complex repetitive units already indicated by the digestion with Asp 718I.

We then cloned the 0.33 kb fragments, corresponding to the repetitive unit of the putative satellite S1b and the 0.39 and 0.5 kb fragments, corresponding to the major fragment and the entire repetitive unit of the putative satellite S1a of *R. dalmatina*. Clonings of the bands of about 0.39 and 0.5 kb, obtained after digestion with Asp 718I (Fig. 3), were performed in the corresponding site of the pTZ19R plasmid and the positive clones were subsequently sequenced. This work was carried out on three clones (RD7, RD10 and RD70) for the largest fragment, and two clones for the entire repeating unit (clones RD83 and RD84). The two sequences showed the same length as the corresponding cloned fragments from *R. italica* and, that is, 385 bp and 494 bp, respectively. As shown in Fig. 4, all five clones show very high homology to each other and, as expected, are also highly homologous to RiS1a.

Fig. 3 Elettroforesi preparativa in gel di agarosio del DNA di *R. italica* (corsia 1) e di *R. dalmatina* (corsia 2) digerito con Asp 718 I. La migrazione dei frammenti è indicata sulla dx.

		50	100
Cons. 494	ACCTAGTGTGACCGGTAAATCGGTAAATTCCTGGTAAAAGGTCTAGAACTTCAAAATTTTGATATGCTCCRGTTGCTATTTCAGGAAGCTGCTCCCCAA		
RD 84	-----T-----G-----		
RD 83	-----A-----A-----G-----		
RD 7	-----A-----A-----		
RD 10	-----A-----A-----		
RD 70	-----T-----A-----C-----		
		150	200
Cons. 494	GTTTGAAGTCTCTAGCACCTTCCGTTCAAAAGTTATAGCCCAAAAACCAATTTACACCAATTTAGGCTGATGTCCCAAAACACCCAMCTTGGACGGSC		
RD 84	-----G-----G-----C-----A-----CT-----T-----C-----		
RD 83	-----G-----A-----C-----		
RD 7	-----G-----A-----A-----C-----		
RD 10	-----C-----C-----G-----		
RD 70	-----C-----A-----G-----		
		250	300
Cons. 494	AGTTCTCCCGAACCATAACHGATAGCGACTTGCTTTTTCGCCACATGTGTAGCCCCATATGTCTGAACMGAMCAAGCCAAGTTTGGGGTCCAAGGGTCC		
RD 84	-----AC-T-----A-----C-----G-----C-----C-----		
RD 83	-----A-----C-----T-----A-----C-----T-----		
RD 7	-----C-----C-----T-----C-----CA-----		
RD 10	-----A-----T-----A-----A-----C-----		
RD 70	-----A-----A-----T-----T-----A-----A-----		
		350	400
Cons. 494	GATGGCCGTGGAGATATCGCGTCTCAAGCTAACCTGTTCFCCCATAGACTCCCATGTAAACGSTCCATCTTTGGAGGCAAGGTACCGGCCAGCCCTTAG		
RD 84	-----A-----A-----T-----T-----G-----		
RD 83	-----T-----C-----G-----		
RD 7	-----A-----G-----		
RD 10	-----C-----		
RD 70	-----C-----		
		450	494
Cons. 494	GTGCCAGTGACCCACCTTTGGGTAGACATGTAGCYGAGAGTCTCCCTACARATGTGGGTAGTTTGGAGGTCTATRCACCAAAGKCCAGAGGGT		
RD 84	-----C-----G-----A-----G-----T-----		
RD 83	-----T-----A-----C-----A-----G-----		

Fig. 4 Sequenza consenso e sequenze dei cloni RD84 e RD83 dell'intera unità ripetitiva RdS1a e sequenze dei cloni RD7, RD10 e RD70 del frammento maggiore di RdS1a del DNA satellite di *R. dalmatina*.

We carried out the cloning of 0.33 kb fragments, obtained by digestion with the Eco RV enzyme (Fig. 5), in the Sma I site of pTZ19R. Six of the positive clones thus obtained were subsequently sequenced (clones RD11, RD15, RD30, RD37, RD39 and RD41). Also in this case a very high homology was found between the various sequences and the same length equal to 332 bp (Fig. 6). From all these experimental data we have defined the consensus sequences of the repetitive units of 494 and 332 bp of the two satellite DNAs called RdS1a and RdS1b, respectively (Fig. 7). The analysis of these sequences shows the presence of an Eco RV site, a Nde I site, and two Asp 718I sites, in the repetitive unit RdS1a, confirming, as expected, that the 0.5 kb fragments obtained after digestion with Eco RV or with Nde I, they correspond to the repetitive unit of previously cloned RdS1a. The major repetitive unit of *R. italica* (RiS1a) has very high homology with RdS1a (about 96%), even if, as previously mentioned, it has a Stu I site, around position 140, not found in the satellite greater than *R. dalmatina* (Fig. 8). Furthermore, if we compare the two minor satellite DNAs, we see that they differ both in length and the restriction sites present. However, they are highly homologous to portions of their corresponding major satellite DNAs, due to the different deletions that originated them, thus making them offset from each other (Fig. 9). RiS1b is approximately 97% homologous to the corresponding first 281 bp of RiS1a, while RdS1b has high homology with the 122-416 base range of RdS1a. Observing the same figure, it can also be noted that RdS1b presents the first 36 bp not homologous to S1a. This sequence corresponds in an inverted sense to that of 36 bp immediately following. The presence of an extended inverted repeated sequence is characteristic, not being present either in RiS1a and RiS1b or in RdS1a.

Fig. 5 Elettroforesi preparativa in gel di agarosio
del DNA di *R.dalmatina* digerito con Eco RV.

Observing Fig. 2, at the level of the lane containing DNA digested with Asp 718I, it is possible to calculate that the doublets of bands at 0.7-0.8 kb, 1.1-1.2 kb and 1.4-1.5 kb could correspond to repeating units of hybrid satellites containing an intact or shortened copy of RdS1a of 109 bp (the small fragment of RdS1a) and one, two or three copies of RdS1b. Among all these bands, particular attention was paid to those of about 0.7 and 0.8 kb. We attempted to clone both fragments in pTZ19R, but at the moment we have obtained and sequenced only 4 clones of the 0.7 kb fragment. Exactly as expected, according to our predictions, all four clones have a length of 717 bp and their sequence is exactly that of the large fragment of RdS1a plus that of RdS1b. It is therefore logical to expect that the band of about 0.8 kb corresponds to the entire repetitive unit RdS1a + b and has a length of 826 bp.

From the length of this sequence and the analysis of the other hybrid bands at the level of the Asp 718I blot, it was observed that the "ladder" is a succession of different repetitive units consisting of the union of a copy of RdS1a and one or more copies of RdS1b digested at one or both Asp 718I sites of RdS1a. As regards the Eco RV and Nde I blots, it should be emphasised that the identification of the hybrid satellite bands is made difficult by the presence of both these restriction sites in both RdS1a and RdS1b, which, on the other hand, does not happen for Asp 718I. In the latter case, consequently, it is difficult to highlight the hybrid satellites that are digested in monomers of S1a and S1b. Only in the case of less complete digestion (lane 8, Nde I) is it still possible to highlight a certain number of bands corresponding to repetitive units of the hybrid satellites. We carried out a further

analysis by Southern blot, comparing DNA of individuals of *R. dalmatina* from different parts of Europe, to compare the patterns and possibly identify intraspecific variability at the level of the satellite DNA organisation. As can be seen from Fig. 10, the Southern blots of various populations, after digestion with Asp 718I (Fig. 10A) and with Eco RV (Fig. 10B), are practically identical. It should be noted that not only the main satellites RdS1a and RdS1b, but also the hybrid satellites are present in all the populations examined. This represents a clear demonstration that the S1 family of satellites constitutes a real molecular marker of the species.

3.2. Quantisation and chromosomal localisation of the satellite DNA

Quantitative dot-blot analyzes were performed to determine how well represented this satellite DNA family is in the *R. dalmatina* genome (Fig. 11). The S1 satellite DNA sequences are present at a concentration of about 50 fmoles/ μ g of DNA. This value corresponds to about 1% of the genome, that is, half of the value obtained for *R. italica*. From previously performed analyzes it has been calculated that the total genome of *R. dalmatina* contains about 9.7 pg of DNA/nucleus (Odierna, 1989). Based on this value, it was found that the copy number of RdS1a and RdS1b per haploid genome is approximately 1.5×10^5 . We also determined the position on the chromosomes of the two satellite DNAs by FISH with the use of biotinylated probes prepared from RdS1a clones. The samples were obtained from blood cultures of *R. dalmatina* and gave a better response than other tissues which previously presented difficulties. In order not to run into the same type of problem previously encountered with *R. italica*, where there was incomplete labelling of

heterochromatin after denaturation at 72 and 75 °C (Cardone et al., 1997), it was preferred to perform DNA denaturation at 82 °C.

In *R. italica* analyses were carried out with probes coming from both the major and minor satellites; the result was an almost identical marking of the chromosomes. In *R. dalmatina* we used only large satellite probes. This procedure was performed for the different structural organisation of satellite DNAs present in *R. dalmatina* and not found in *R. italica*. This organisation, mostly consisting of hybrid satellites containing both S1a and S1b, made it completely useless to verify the hybridisation with an exclusive probe for S1a. The result was different to the chromosome marking obtained in *R. italica* (mainly centromeric). In fact, in *R. dalmatina*, hybridisation occurred mainly at the pericentromeric level, and, only in some chromosomes (Fig. 12), while in *R. italica* all the chromosomes are marked mainly at the centromeric level (Cardone et al., 1997). This result is in agreement with the quantitative dot-blot data since *R. dalmatina* contains about half of the satellite S1 DNA compared to *R. italica*.

Fig.12 Marcatura fluorescente dei cromosomi mitotici mediante ibridazione in situ non radiattiva da colture di sangue di *R. dalmatina* con una sonda biotinilata preparata da cloni dell'unità ripetitiva RdS1a.

4. Discussion

The digestion of the DNA of *R. dalmatina* with Asp 718I (Kpn I) produced two bands of highly repeated DNA which we subsequently cloned and characterised. The largest fragment corresponds to the entire repetitive unit of 494 bp of the major satellite DNA (RdS1a), while the smaller fragment of 385 bp corresponds to the major fragment of RdS1a produced by digestion. Digestion with Eco RV, in addition to producing a fragment of 0.5 kb corresponding to S1a, gives a fragment of 332 bp, which, once cloned and sequenced, we found to correspond to the repetitive unit of satellite S1b (RdS1b). The high homology of the two satellite DNAs, also proven by cross-hybridisation, was confirmed by the sequence analysis. The repetitive unit of 332 bp of the short satellite (RdS1b) is highly homologous for about 296 bp out of 332 bp to the corresponding portion of the repetition of 494 bp of the long satellite (RdS1a). Further, RdS1b presents the first 36 bp repeated and inverted and this has led to hypothesise its appearance from the major satellite through two subsequent events. RdS1b would have been derived from satellite S1a by deletion of a segment of about 200 bp corresponding to the sequences placed upstream and downstream of the 296 bp sequence. This deletion was followed by the duplication of a 36 bp portion which was reinserted with reverse orientation in the vicinity of the duplicated sequence. In practice, the repetitive unit of RdS1b consists of a central unit of just over 0.25 kb flanked on both sides by an inverted repeat of 36 bp. RdS1a has the same length and high sequence homology as S1a of *R. italica* (RiS1a). This confirms the hypothesis that the satellite S1a is antecedent to S1b and inherited by the two species from a common ancestor.

In *R. italica*, the two restriction enzymes, Eco RV and Nde I, exclusively cut the satellite S1a, while, in *R. dalmatina* it is Asp 718I which makes a discriminating cut between the major and the minor unit. Since RdS1b does not present restriction sites for this enzyme, it was thus possible to highlight fragments of different sizes containing in addition to the satellite S1a, also one or more copies of the S1b satellites, which we have defined as hybrid S1 satellites. However, the presence of two Asp 718I sites in the RdS1a repeating unit resulted in the production of double bands for each repeating unit of the hybrid satellites, making Southern blots difficult to interpret. The presence of additional hybrid bands in *R. italica* was explained by the formation of repetitive units obtained from various combinations of the two satellite DNAs. In *R. dalmatina*, between the identified hybrid bands, only the minor of the hybrid satellite S1a + b has been sequenced at the moment. This band, present in the blots of Asp 718I, corresponds to a fragment of 717 bp which, as expected, corresponds to the repetitive unit of the satellite RdS1a + b (826 bp) deleted of the fragment Asp 718I less than RdS1a (109 bp). The other double bands present in the DNA digested with Asp 718I should almost certainly correspond to repetitive units of satellites RdS1a + 2b and RdS1a + 3b. Fragments corresponding to one or more of these repetitive units are also evident after digestion with Eco RV and Nde I. The only hybrid band that is difficult to interpret appears to be that of about 0.73 kb, present in the DNA digested with Eco RV.

From the analysis of the genomic DNA of various populations of *R. italica*, it has been seen that the amount of hybrid satellites is low and extremely variable between individuals, even in the same population. This strong intraspecific

variability, on the other hand, was never detected in the experiments between specimens of *R. dalmatina* from different locations. In this species, a significant fraction of the satellites S1a and S1b appear to be present in hybrid satellites. Therefore, due to all these data, it is possible to identify various types of satellite DNA organisation in *R. dalmatina*. The first consists of the arrangement of the repetitive units of the RdS1a satellite in tandem, another organisation is in the tandem structure of RdS1b. In addition to these simple arrangements, there are hybrid satellite DNAs formed by repeating units containing one or more copies of both S1a and S1b. Our data suggest the existence of at least four different types of hybrid repeating units. While in *R. italica* the S1 hybrid satellite DNAs are present in small or very variable quantities, in *R. dalmatina* they seem to be present in high quantities in all the populations examined.

4. Conclusions

In conclusion, from previous hypotheses it seems that all European brown frogs are derived from a common ancestor of the Pliocene (Bolkay, 1985, Picariello et al., 1990), from which probably derives the satellite S1a present in all these frogs. This family of satellite DNA, typical of brown frogs, shows, as shown by our study, species-specific variations in both structure and sequence. We believe that the characterisation of these satellites should provide if extended to other brown frog species, important information on the evolution and systematics of this whole group of amphibians. Further research, therefore, is needed to definitively shed light on the phylogeny of the species group of European brown frogs.

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