

Microsatellites from *Rana dalmatina*



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Introduction

Rana dalmatina is widely distributed in Europe and its southwest distribution limit is the Iberian peninsula. There, the species has a reduced range in the southwest of the Pirineos mountain range, limited to the provinces of Basque country and Navarra.

The populations are very threatened mainly by the loss of its habitat, that coincide with high rates of human activities, because takes up the valleys. The optimum habitat is a mosaic with oaks forest (*Quercus robur* and *Q. pyrenaica*) and pastures, with vegetate ponds where breeding. The surviving populations are separated by large barriers as a result of urbanization, agricultural fields, roads, etc. The migration between these populations is very difficult or impossible, so the populations are isolated and its structure, subdivision, and genetic diversity remains unknown.

The study of the species phylogeography and of the population genetics in the Iberian peninsula is very useful to protect the remaining populations.

A study using polymorphic microsatellites will allow assessments of *R. dalmatina* genetic diversity, problems of inbreeding depression in the subpopulations, gene flow and the testing of whether metapopulations exist.

Methods

Microsatellites from *Rana dalmatina* have been isolated *de novo*. Fresh tissue samples from the Iberian populations were used to extract total genomic DNA using the Phenol-Chloroform extraction method.

High quality genomic DNA, measured in agarose gel after electrophoresis, was fragmented using the restriction enzyme Mbo1, which recognize and cut a sequence of 4bp, to obtain DNA in size range 300-700 bp.

These fragments are joined to specific linker (Saul A and Saul B) and amplified together using the Polymerase Chain Reaction.

The first hybrid selection was made in this step, looking for the next microsatellite sequences: GT/AC, GATA/TATC, AG/CT and GACA/TGTC. The DNA is hybridized with the mentioned repeat containing probes 5'biotinylated and bound to streptavidin coated beads. After several washes to remove non-specific binding, the selected DNA was amplified again by the PCR. Linkers were removed using again the endonuclease Mbo 1.

The dephosphorylated plasmid pUC19 was digested with another restriction enzyme (BamH1) what generate compatible ends with the ends from the first enzyme Mbo 1. Then DNA fragments were incubated with it, in the appropriate conditions and in presence of T4 DNA ligase, and linked into the plasmid.

The plasmids with the insert DNA were included in competent DH5 α cells of *Escherichia coli* and cloning in LB/agar with ampicillin and X-gal to select the cells using the ampicillin resistance locus in the plasmid, and the method white/blue based on the lac operon expression. White colonies were transferred by picking single colonies and ordering them in new arrayed plates.

After blotting bacterial colonies onto nylon membranes (Hybond-N membrane), probes synthesized *de novo* and labelled by radioactive ³²P were labelled, in hybridisation solution.

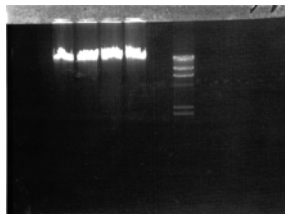
Following identification of clones which had included a sequence with microsatellites (phosphorimager screen), the DNA was extracted from the bacteria using Quiagen kit, was sequenced, the microsatellites were found and specific primers were designed using the computer program PRIMER3.



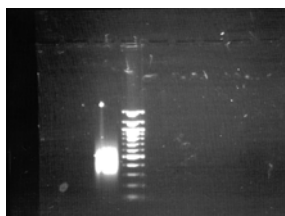
Rana dalmatina from Spain



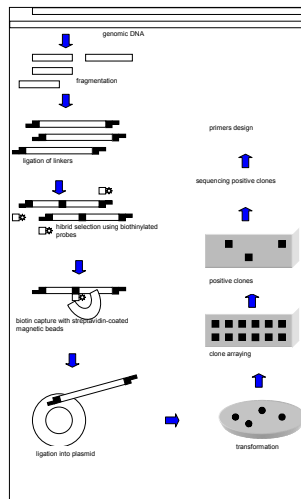
Populations of *Rana dalmatina* from Iberian peninsula used in this study



Size and purity of DNA preparation in agarose gel



Result after the PCR of the hybrid-selected DNA



Schematic representation of the method for microsatellite isolation

Finally, DNA from 10 frogs was purified (4 from Izki population in the south extreme of its Iberian distribution, 3 from Ultzama population, in the east extreme, and 3 from Amurrio population, in the west extreme), and the microsatellites were amplified by PCR using the design primers, nucleotides and labelled ATP. The samples were put and ran in a polyacrylamide gel and the result was revealed. The polymorphic microsatellites have more than one different allele.

Furthermore three primers from *R. temporaria* and one from *R. latastei* were tested in the samples.

Results and perspectives

We have sequenced twenty microsatellites from the insert DNA in the selected bacteria and designed the primers to amplify its. Using the DNA from the Iberian larvae we tested the designed primers and the polymorphism of the microsats, nine of them are polymorphic, and three monomorphic. Eight times we designed new primers because we had not any result after the amplification using the old primers.

The rest of microsatellites, will be tested again this year in order to obtain good results. Furthermore we have tested one from *R. latastei* and three from *R. temporaria*. The first is polymorphic in our samples, but no the rest.

We will test the microsatellites in European populations where, in principle the polymorphism will be greater.

After this, we would like to study the populations in the Iberian peninsula, its genetic diversity and gene flow, what provide new perspectives and points of view to protect it.

	Polymorphic	Monomorphic
Rtemp1		X
Rtemp5		X
Rtemp9		X
RtatCa17	X	
Rdal-1	X	
Rdal-2		X
Rdal-3	X	
Rdal-4		X
Rdal-5	X	
Rdal-6	X	
Rdal-7	X	
Rdal-8		X
Rdal-12	X	
Rdal-18	X	
Rdal-19	X	

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