Role of molecular markers in environmental studies on the example of the endangered species *Cistus heterophyllus*

Papel de los marcadores moleculares en los estudios ambientales: el ejemplo de la especie amenazada *Cistus heterophyllus*

M. Pawluczyk*, J. Weiss, M. Egea-Cortines

Genetics, Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena. 30202, Cartagena, Spain

Abstract

This study aims to analyse a set of molecular markers for tracing hybridization events in the population of an endangered species from the Cistaceae family, *Cistus heterophyllus* subsp. *carthaginensis* limited to only one natural population in the north-eastern Spain. In this population individuals with wild type and hybrid phenotypes, described before in Africa as *C.* × *clausonis,* co-occure, suggesting hybridization events between the endangered population and the locally abundant *Cistus albidus*. We applied plastid DNA and internal transcribed spacer (ITS) region of the ribosomal DNA as markers in the aforementioned population. We observed heteroplasmy for *rpoB* and *rpoC1* plastid genes in *C. heterophyllus* and the local *C.* × *clausonis*, but not in *C. albidus*. The ITS region was analysed in geographically isolated populations of *Cistus heterophyllus*, *Cistus albidus* and possible hybrids of these two species. Depending on the individual and population, *C.* × *clausonis* phylogenetically resembles more either *Cistus heterophyllus* or *Cistus albidus* and it might be related to the homogenization of variation between repeat types through concerted evolution.

Keywords: Cistus; molecular markers; plastid genes; ITS.

Resumen

Este estudio tiene como objetivo analizar un conjunto de marcadores moleculares para el rastreo de eventos de hibridación en la población de una especie en peligro de extinción de la familia Cistaceae, *Cistus heterophyllus* subsp. *carthaginensis* limitada a una sola población natural en el noreste de España. En esta población coocurren individuos con fenotipo silvestre y fenotipos híbridos, descritos antes en Africa como *C.* × *clausonis*, lo que sugiere eventos de hibridación entre esta población en peligro de extinción y una especie localmente abundante, *Cistus albidus*. Hemos aplicado marcadores plastídicos y la region internos inter-espaciados (ITS) de la ADN ribosómico como marcadores para su aplicación en la población mencionada. Observamos heteroplasmia en *C. heterophyllus* y *C.* × *clausonis* local, pero no en *C. albidus*. La región de ITS fue analizada en poblaciones geograficamente aisladas de *Cistus heterophyllus*, *Cistus albidus* y posibles híbridos entre estos dos especies. Depnediendo de individuo o población, *C.* × *clausonis* filogenéticamente parece más a *Cistus heterophyllus* o *Cistus albidus* y problamente está relacionado a la homogenización de variación por evolution concertada.

Palabras clave: Cistus; marcadores moleculares; genes plastídicos; ITS.

* E-mail: marta.pawluczyk@gmail.com

1. INTRODUCTION

Molecular markers are a very powerful tool in environmental studies. They can be especially useful when investigating hybridization processes. However, it is not an easy task to find proper markers for rare species. In the situation of lack of sequence information about their genomes, the only solution are universal markers already described for other organisms. The most common plant universal markers are regions known as ¨DNA barcodes¨. Application of plastid noncoding DNA regions (*rbcL, trnK-matK*, *rpoB, rpoC1*) and plastid intraspecific regions (*trnL-F, trnH-psbA)* were already described by Chase [1] and Kress & Erickson [2,3]. It was suggested that these chloroplast markers could provide preliminary information of the extent and nature of population divergences and support comparative studies on population diversity [4]. Nuclear ribosomal DNA regions, in particular the internal transcribed spacer (ITS) region, are useful plant universal marker, especially in detecting hybrid relationships of generic and interspecific nature in flowering plants [5,6].

Cistus heterophyllus subsp. *carthaginensis* is an endangered species from the Cistaceae family. The unique natural population in Parque Regional de Calblanque, Monte de las Cenizas y Peña del Águila (Murcia) consists of 22 plants with two distinct morphologies: 12 individuals resemble what would be a pure *C. heterophyllus* subsp. *carthaginensis* type whereas 10 plants show an intermediate phenotype similar to hybrids described as *C.* × *clausonis* (*C. heterophyllus* × *C. albidus*) from northern Africa. As there are no exhaustive molecular studies for the *Cistus* family, we applied plastid and nuclear ITS markers in order to examine the hybridization events in the endangered species *C. heterophyllus* subsp. *carthaginensis.*

2. MATERIALS AND METHODS

2.1 Sampling of plant material

For the plastid marker experiment, plant material of *C. heterophyllus* (3 populations)*, C. albidus* (5 populations) and *C.* × *clausonis* (2 populations) was collected. For the ITS marker studies, 114 individuals were collected: 25 individuals of *C. albidus* (10 populations), 70 individuals of *C. heterophyllus* (21 populations) and 17 individuals of *C.* × *clausonis* (4 different populations).

2.2 DNA extraction, cloning and sequencing

Total genomic DNA was extracted from plant material using a commercial kit according to the instruction manual. Selected DNA fragments were amplified under PCR conditions suitable for each primer. The primers used in this experiment have been described previously: plastid markers [2,3] and ITS [7]. PCR products were sequenced on an Abi Prism 3130XL Genetic Analyzer (Applied Biosystem, Foster City, CA, USA).PCR product for plastid markers were cloned before sequencing.

2.3 Real-time PCR, melting analysis for *rpoB* and *rpoC1* genes and identification of polymorphisms by restriction digestion

The loci *rpoB* and *rpoC1* were analyzed with the Mx3000P Q-PCR System using the SYBR Premix ExTaqTM (Takara Biotechnology, Dalian, China). To differentiate between *rpoC1* alleles A and B, PCR products of all sampled individuals were digested with ClaI restriction enzyme (Fermentas, Hanover, MD, USA).

3. RESULTS AND DISSCUSION

3.1 Plastid markers

Noncoding DNA regions (*rbcL, trnK-matK*) were found as not variable enough to be informative in closely related individuals. Intraspecific regions (*trnL-F, trnH-psbA*) presented a high rate of evolutionary changes as indicated by their high variability. However, we found these markers as not sufficiently stable to give reliable information for the identification of wild type and hybrid individuals. Surprisingly, we observed heteroplasmy for *rpoB* and *rpoC1* genes in *C. heterophyllus* and the local *C. × clausonis*, but not in *C. albidus* (Fig.1).

We found two distinct alleles of *rpoB*, one present in all species and a second present only in *C. heterophyllus* and the local *C. × clausonis*. We also detected two alleles of *rpoC1*, one common to all species analyzed and a second present only in the local *C. × clausonis*. Our results show that there is a distinctive *rpoB* allele common to *C. heterophyllus* and *C. × clausonis* from Africa and Europe. The unique *rpoC1* allele found in the local *C. × clausonis* directs to a different origin of this small population, indicating that it is not a hybrid originating from *C. albidus* or *C. heterophyllus* currently present in this location.

3.2 ITS marker

The cladogram constructed based on the ITS sequence analysis with the outgroup *C. ladanifer, C. monspeliensis* and *C. laurifolios* surprisingly shows that the ITS sequence amplified from *C. heterophyllus* individuals collected in Llano de Beal did not place them on a common branch with another *C. heterophyllus* plant, but rather together with one of the hybrids (Fig.2). Another hybrid branched closely to a specific group of plants of *C. albidus* from Llano de Beal and a third hybrid took an obvious intermediate position between *C. heterophyllus* and *C. albidus* on a separate branch.

4. CONCLUSIONS

Among the plastid markers, we found the genes *rpoB* and *rpoC1* useful in differentiation between *C. heterophyllus* and *C. × clausonis* from *C. albidus.* The ITS marker analysis shows that, depending on the individual and population, *C. × clausonis* phylogenetically resembles more either *Cistus heterophyllus* or *Cistus albidus.* This situation might be a result the homogenization of variation between repeat types through concerted evolution.

5. ACKNOWLEDGMENTS

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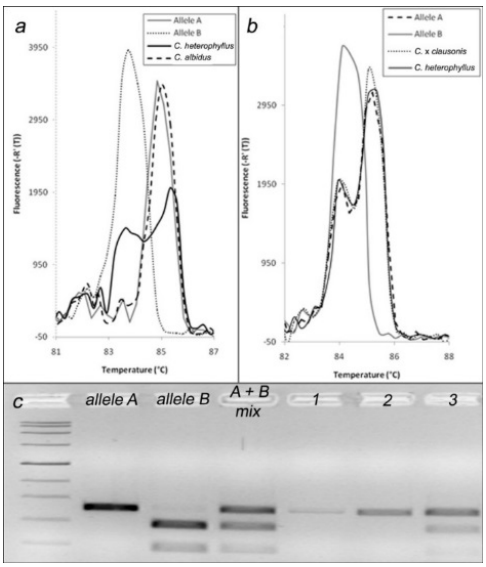

Figure 1. Melting curve qPCR analyses from of allele A and B of the *rpoB* gene present in *C. heterophyllus* individual. In *C. albidus* only allele A is present; b. Melting curve for *rpoC1* gene - allele A with double melting peak at 84.10 °C and 85.20 °C and allele B at 84.10 °C. Curve for *C. heterophyllus* without allele B and curve for *C. × clausonis* subsp. *carthaginensis* containing allele B; c. CAPS marker – *ClaI* enzyme digested only samples containing allele B or mix of two alleles. Samples containing only allele A remain undigested

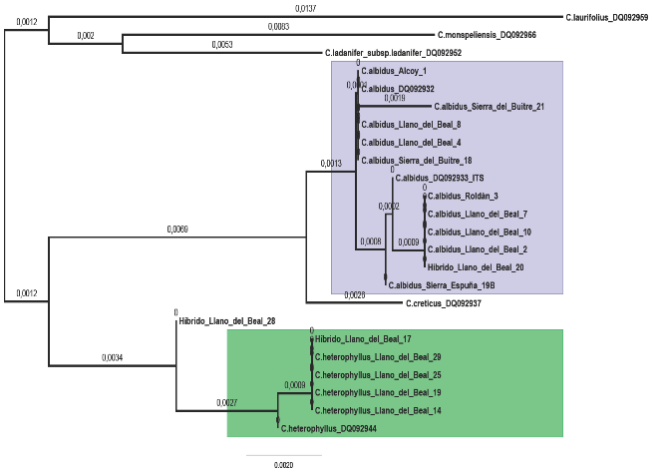

Figure 2. Cladogram of *C. heterophyllus* subsp. *carthaginensis, C. albidus* and *C. × clausonis* based on ITS region.