Microsatellite cross-species amplification in the genus Centaurea (Compositae)

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Abstract
Microsatellite cross-species amplification in the genus Centaurea (Compositae).- Microsatellites are widely used for population genetic studies although the development of these species-specific markers is costly and time-consuming. One strategy for saving time and money is the use of markers developed for one species (source species) in a different species (target species). This is known as cross-amplification. In the present work, two sets of microsatellites are used to test their transferability to six narrow endemic Centaurea species: i) 16 nuclear loci previously published for three congeneric species and ii) 10 universal chloroplast markers designed from Nicotiana tabacum sequences. Seventeen of the 26 markers tested were transferable and 14 of them were also polymorphic and therefore useful for future works. Nuclear markers were more variable and thus more informative than chloroplast markers. Interspecific amplification performed better for the nuclear loci developed for different Centaurea species than for the universal chloroplast markers developed for Nicotiana tabacum. Likewise, transferability was more successful for the species from sect. Phalolepis than for sect. Lepteranthus. Therefore, our results support the idea that the success of the cross-amplification is influenced by the evolutionary distance between the target and the source species.

Key words: chloroplast microsatellites; conservation; cross-amplification; interspecific amplification; narrow endemics; SSR; transferability; universal markers.

Resumen
Amplificación cruzada de microsatélites en el género Centaurea (Compositae).- Los microsatélites se usan habitualmente en estudios de genética de poblaciones aunque su desarrollo es un proceso caro y largo dado su elevada especificidad. Una estrategia que permite ahorrar tiempo y dinero es la llamada amplificación cruzada que consiste en amplificar el DNA de una especie determinada (especie objetivo) usando marcadores que han sido diseñados para una especie diferente (especie fuente). En este trabajo se ha realizado un ensayo sobre amplificación cruzada usando seis endemismos del género Centaurea y dos conjuntos de microsatélites: i) 16 marcadores nucleares desarrollados para tres especies congenéneas y ii) 10 marcadores cloroplásticos universales diseñados para Nicotiana tabacum. Diecisiete de los 26 marcadores evaluados resultaron transferibles, de los cuales 14 fueron polimórficos siendo así útiles para futuros trabajos. Las regiones nucleares mostraron más variables y por lo tanto más informativas que las cloroplásticas. La amplificación cruzada funcionó mejor para los marcadores nucleares específicos de Centaurea que para los cloroplásticos universales específicos de Nicotiana tabacum. A su vez, se obtuvo mejor resultado para las especies de la sección Phalolepis que para las de la sección Lepteranthus. En consecuencia, nuestros resultados apoyan la idea de que el éxito de la amplificación cruzada está estrechamente ligado a la distancia evolutiva entre especie fuente y especie objetivo.

Palabras clave: amplificación cruzada; amplificación interespecífica; conservación; marcadores universales; microendemismos; microsatélites cloroplásticos; SSR.
INTRODUCTION

Single Sequence Repeats (SSR), also known as microsatellites, are widely used to investigate genetic variability and structure both at population and species level. These genetic markers are tandemly repeated units from one to six nucleotide in length distributed evenly throughout the genome and present in almost every organism so far studied (Hancock, 1999). Their main advantages are i) high polymorphism derived from a high mutation rate, ii) codominant inheritance and iii) multiallelic nature.

The process of SSR isolation is expensive and time consuming since they usually have to be designed and optimized ex novo for each species separately. To avoid these drawbacks, the use of primer sequences developed for one species (source species) into others (target species) is desirable in terms of cost-efficiency. Ideally, the development of universal primers would minimize both time and costs necessary to carry out microsatellite works. The main weakness of this strategy is the low variability recovered in most cases because universal primers often amplify loci in coding regions. Different studies have used this approach mainly for chloroplast DNA (e. g. Weising & Gardner, 1999; Chung & Staub, 2003) and Expressed Sequence Tags (EST, e. g. Peakall et al., 1998; Heesacker et al., 2008). Regarding chloroplast microsatellites, Ebert & Peakall (2009) state that “when genus-specific cpSSR primers are available, cross-species amplification can often be fruitful”. Nevertheless, they point out that “while potentially useful, universal cpSSR primers at best provide access to only a small number of variable markers”. In addition, an important limitation of cpDNA markers is that they constitute a single linkage group. EST-derived markers come from transcribed regions of the genome, so they are likely to be conserved across a broader taxonomic range than markers derived from total genomic DNA. Pashley et al. (2006) found that this kind of marker was more than 3 times as transferable across species than were the anonymous SSRs derived from genomic DNA (73% vs. 21%, respectively). However, according to Kantety et al. (2002) the frequency of SSR-containing sequences in plant-derived EST databases is only around 2% and 5%.

Therefore, as a starting point for a population genetic study in a given species, it is worth testing markers previously developed for congeneric plants if these are available, in few individuals. If they are useful, there is no need to look for new loci, saving time and money. If amplification fails or these loci are monomorphic, development of specific microsatellites will be necessary.

According to Barbará et al. (2007) the success of the cross-amplification is highest in species with long generation times, mixed or outcrossing breeding systems, and when genome size in the target species is small compared to the source. In addition, negative association between evolutionary distance and cross-species amplification success has been reported by several authors (e. g. Primmer et al., 1996; Eujayl et al., 2004; Saha et al., 2004; Guo et al., 2006). This phenomenon is explained primarily because polymorphisms in sequences flanking repeats increase as phylogenetic distances increase. As an example, Heesacker et al. (2008) found that of the 466 markers developed for Helianthus annuus L. successful cross-amplification was achieved for 88.6% of the loci in sunflower wild relatives H. anomalus S. F. Blake, H. argophyllus Torr. & A. Gray, H. deserticola Heiser, H. paradoxxus Heiser and H. tuberosus L. whereas only around 14% of the loci amplified alleles from distantly related species such as Carthamus tinctorius L. and Lactuca sativa L.

Taking into account all these considerations, in the present work we will test the transferability of 26 microsatellite markers previously developed for Nicotiana tabacum L., Centaurea corymbosa Pourr., C. diffusa Lam. and C. stoebe L. to six narrow endemic Centaurea L. species from two different sections, Lepteranthus (Necker) DC. and Phalolepis (Cass.) DC. (sensu Dostál, 1976).

Our goals are:

To investigate if the cross-amplification performs better with phylogenetically closer species using microsatellite nuclear markers.

To verify usefulness of SSR markers within the Greek group of narrow endemics from sect. Phalolepis.

MATERIALS AND METHODS

Plant material

Studied material comprises six narrow endemic Centaurea species.

Centaurea emigrantis Bubani is a perennial herb from sect. Lepteranthus restricted to the Pre-
Phalolepis in Greece is composed of Centaurea chrysocephala Phitos & T. Georgiadis, C. heldreichii Halácsy, C. litochorea T. Georgiadis & Phitos, C. messenicolasiana T. Georgiadis, G. Dimitrellos & Routsi and C. princeps Boiss. & Heldr.

Centaurea chrysocephala is a biennial or perennial plant known from a few localities in Central Greece, where it forms small populations. It prefers rocky places and cliffs but can also be found in stony places, road margins and walls, on limestone, sandstone, serpentine and conglomerate substrates. It is a diploid with 2n = 18 (Routsi & Georgiadis, 1996) and has been characterized as R (Rare) by Constantinidis (2009) and is possibly extinct at its type locality (Strid & Franzen, 1981). Therefore we consider that the report by Strid & Franzen (1981) is inaccurate and should be discarded.

Centaurea princeps is a local endemic only known from Mount Timfristos of Central Greece. It is a biennial growing in limestone cliffs and scree, at an altitude of 1100-1850 m. It is taxonomically related to C. chrysocephala and C. messenicolasiana and diploid, with 2n = 18 (Garcia-Jacas, unpublished data). It is a threatened plant, characterized as VU (Vulnerable) by Georgiadis & Dimitrellos (2009).

Localities of the material used and voucher specimens information are listed in Table 1. In addition, a picture of each species is provided in Fig. 1.

**DNA isolation and microsatellite loci**

Genomic DNA was extracted from dried leaf tissue using the CTAB method by Doyle & Doyle (1987) as modified by Cullings (1992) and Tel-Zur et al. (1999). In order to increase the detection of polymorphism in this preliminary screening, individuals within a species were selected from different populations when possible. Ten individuals each from a total of 15 wild populations representing the six species were sampled (Table 1).

In this cross-amplification assay, two sets of microsatellites were used: i) nuclear markers: seven SSR loci previously developed for C. corymbosa (Fréville et al., 2000) and nine SSR loci previously developed for C. diffusa and C. stoebe (Marrs et al., 2006), all three species from sect. Centaurea [=Acrolophus (Cass.) DC.] and ii) 10 cpDNA markers designed for Nicotiana tabacum (Weising & Gardner, 1999). See Table 2 for details. All SSR loci were amplified using FAM, NED, PET and VIC fluorescently labeled forward primers as explained in López-Vinyallonga et al. (2010). Different profiles were used for the amplification following the conditions established for each locus in the original publications (Table 2). The six target species were amplified with the nuclear markers while five species were amplified with the chloroplast set (C. emigrantis was not included in this assay). Genotyping was performed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Fos-
Table 1. Voucher information and sources of material of the six species included in this work.

| Species                        | Voucher                                                                                           |
|-------------------------------|---------------------------------------------------------------------------------------------------|
| C. chrysocephala Phitos & T. Georgiadis | Greece, Nomos Trikalon, Eparchia Kalampakas, Mt. Kerketion Oros, c. 1 km E of the village of Klinovo, 39° 39’ 59.32” N, 21° 28’ 19.26” E, Garcia-Jacas, Karamplianis & Susanna 2742 (BC).  
Spain, Lleida, Òger, near Corçà, road margins, 42° 2’ 7.11” N, 0º 41’ 66” E, López-Alvarado & Figueroa s. n. (BC). |
Figure 1. Studied species. (A), Centaurea messenicolasiana; (B), C. princeps; (C), C. litochorea; (D), C. heldreichii; (E), C. chrysocephala; (F), C. emigrantis.
ter City, CA, USA) at the Interdisciplinary Center for Biotechnology Research (ICBR) facility at the University of Florida. Fragment analysis was performed with GENEMARKER 1.5 (SoftGenetics, LLC, State College, PA) software using 600 LIZ size standard (Applied Biosystems), and data was scored manually. Tables 3 and 4 show the monomorphic or polymorphic nature of each marker for each species considered.

### Data analysis

The software GenAlEx6 (Peakall & Smouse, 2006) was used to perform the genetic diversity analysis for the six Centaurea species included in this transferability assay. The statistical parameters computed were: the number of alleles; the number of private alleles (PA); the Shannon’s Information Index (I) and the observed heterozygosity ($H_o$) which represents

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**Table 2.** Marker information: locus names, bibliographic references, repeat motifs, size ranges of PCR products and number of alleles observed in the original publications.

| Loci  | Repeat | Size (bp) | No. of alleles | Reference     |
|-------|--------|-----------|----------------|---------------|
| CM26  | (TG)$_4$(TA)$_3$ | 133–172   | 22             |               |
| CD37  | (CA)$_6$    | 154–176   | 7              |               |
| 21CM36| (CA)$_4$(TA)$_4$(TG)$_6$ | 187–244  | 6              |               |
| 38CM22| (GA)$_2$(AA(GA)$_8$ | 289–344  | 25             |               |
| CD9   | (CA)$_7$    | 301–325   | 9              | Marrs et al. (2006) |
| 42CM27| (TG)$_4$    | 155–175   | 11             |               |
| CM15  | (GT)$_6$    | 185–220   | 10             |               |
| 25CM6 | (CA)$_6$    | 255–272   | 7              |               |
| CM17  | (AC)$_6$    | 379–430   | 16             |               |

**Table 3.** Nuclear loci tested. Allele number per locus for each species, total number of alleles per each locus and per each species is given. -: failed reaction.

| Species                  | CM26 | CD37 | 21CM36 | 38CM22 | CD9 | 42CM27 | CM15 | 25CM6 |
|--------------------------|------|------|--------|--------|-----|--------|------|-------|
| C. chrysocephala         | -    | 3    | -      | 5      | -   | 6      | -    | -     |
| C. emigrantis            | -    | -    | -      | -      | -   | -      | -    | -     |
| C. heldreichii           | -    | 1    | -      | 1      | -   | 4      | -    | -     |
| C. lithocephra           | -    | 2    | -      | 4      | -   | 11     | -    | -     |
| C. messenecolosiana      | -    | 3    | -      | 3      | -   | 3      | -    | -     |
| C. princeps              | 2    | 2    | -      | 2      | -   | 6      | -    | -     |
| Total                    | -    | 5    | 7      | 7      | -   | 12     | -    | -     |
the percentage of heterozygous individuals in a given sample. All these parameters were calculated both for each species and for each polymorphic locus ($H_o$ was not computed for the chloroplast markers because of the haploid nature of this genome). In addition, the percentage of polymorphic loci was computed at the species level. Given the low polymorphism of the chloroplast loci tested (Tables 4 and 5), the genetic diversity statistics at species level where computed using only the nuclear set of markers. Since the chloroplast markers were not amplified for C. emigrantis, the diversity values for these loci were computed on the basis of five species instead of six.

RESULTS

Seventeen (65.4%) of the 26 SSR markers tested produced consistent cross-amplification in Centaurea and accordingly they were considered as transferable (Tables 3 and 4). Slightly different percentages of marker transferability were recorded for each type of markers; 11 out of 16 nuclear markers tested were transferable (68.75%), whereas six out of 10 chloroplast loci assayed were transferable (60%). Cross-amplification details for every marker are indicated in Tables 3 and 4.

Within the 17 transferable markers 14 (82.35%) were polymorphic while 3 (17.65%), all of them from the chloroplast genome, were monomorphic. At the species level, the overall percentage of polymorphic loci for the transferable nuclear markers was 92.5% ranging from 100% for C. chrysocephala, C. emigrantis, C. litochorea and C. princeps to 75% for C. heldreichii and C. messenicolasiana to 50% for C. heldreichii and C. messenicolasiana to 16.67% for C. litochorea (Table 5).

All the 11 nuclear loci amplified were polymorphic (Table 3). Within them, three markers (12B1, 13D10 and 28A7) provided good amplification products for the six species tested, five markers (CD37, 38CM22, 42CM27, 17E3 and 21D9) provided good amplification products for five species and three markers (21CM36, CM17 and 13B7) provided good amplification products for just one species. The nuclear locus showing the highest number of alleles was 21D9 (17 alleles amplified from five species) while the lowest number was recovered for locus 13B7 (three alleles, although it is worthy to highlight that it was successfully amplified only for C. emigrantis). The species with the highest number of alleles for the nuclear markers was C. litochorea, with 50 alleles from a total of eight markers. In contrast, the species with the lowest number of alleles for the nuclear markers was C. heldreichii, with 27 alleles from a total of eight markers. See Table 3 for details.

Among the six chloroplast loci amplified, with the five species tested giving amplification products, three of them were polymorphic with three alleles each (ccmp3, ccmp4 and ccmp5) and the other three were monomorphic (ccmp2, ccmp6 and ccmp7). The species with the highest number of alleles for the six chloroplast markers together were C. chrysocephala, C. heldreichii, C. messenicolasiana and C. princeps with nine alleles, while the species with the lowest number of alleles was C. litochorea, with seven alleles. See Table 4 for details.

Regarding the genetic diversity parameters at species level, the observed heterozygosity ranged from 0.55 (C. chrysocephala) to 0.30 (C. heldreichii) and the Shannon’s Information Index ranged from 1.45 (C. chrysocephala and C.
DISCUSSION

As stated by many authors (e.g. Primmer et al., 1996; Eujayl et al., 2004; Saha et al., 2004; Guo et al., 2006; Heesacker et al., 2008), cross-amplification is expected to work better for phylogenetically close species, even more when source and target species are congeneric. This can be explained because the similarities in the flanking regions of the microsatellite, where the PCR primers bind to the DNA, are higher in phylogenetically close species than phylogenetically distant species. In our assay, the amplification process was slightly less successful for the set of universal chloroplast markers developed for *Nicotiana tabacum*, than for the set of nuclear markers developed for different *Centaurea* species (60% and 68.75% of the loci amplified respectively). Therefore the aforementioned trend is detected in our data but since the source species of the nuclear markers were from genus *Centaurea* and the chloroplast markers were developed for a phylogenetically very distant species, we expected higher differences in the transferability success. Yet, although the percentage of amplified loci is similar for both genome types, the percentage of polymorphic loci is strongly higher for the nuclear markers (92.5%) compared to the chloroplast markers (36.67%). The lower variability detected in the cpDNA is in keeping with the slow substitution rate and low rate of structural evolution in the plastid genome relative to the nuclear genome. Moreover, the PCR primers amplifying the chloroplast markers used here were designed in conserved regions of the genome in order to be transferable to many plant species (Weising & Gardner).

The relationship between evolutionary proximity and success of SSR transfer between source and target species was also tested for *Centaurea* at sectional level using nuclear markers. The most recent molecular studies in the genus *Centaurea* (Garcia-Jacas et al., 2006; Suárez-Santiago et al., 2007) have revealed that sections *Centaurea*, *Phalolepis*, *Willkommia* G. Blanca, *Jacea* (Mill.) DC. and *Lepteranthus* form a natural group, where the first three sections constitute a monophyletic clade clearly separated from...
sections Jacea and Lepteranthus. The source species of the markers used in this transferability assay (C. corymbosa, C. diffusa and C. stoebe) belong to sect. Centaurea. As expected, cross-amplification performed better for C. heldreichii, C. messenicolasiana, C. chrysocephala, C. princeps and C. litochorea, all of them from sect. Phalolepis (eight out of 16 nuclear loci amplified) than for C. emigrantis from sect. Lepteranthus (six out of 16 nuclear loci amplified).

Regarding the usefulness of the markers testing the number of alleles, the percentage of polymorphic loci for each species and all the diversity parameters except the number of exclusive alleles were higher for the nuclear loci. As previously explained, the genetic diversity statistics were computed over six species for the nuclear loci and for five species for the chloroplast loci which could bias slightly downwards the values of these parameters for the latter. Nevertheless, we consider that this possible bias can be negligible for the purposes of the present work. In addition, all the nuclear loci tested were polymorphic while 50% of the chloroplast markers successfully amplified were monomorphic. Again, this denotes that nuclear markers are more variable than chloroplast markers for all the target species assayed regardless of their phylogenetic distance with respect to the source species and therefore they are more informative and suitable for future works.

As far as genetic diversity is concerned, direct comparison of section Lepteranthus and section Phalolepis through the computed statistics is precluded because the SSR markers amplified are not the same for both sections. Moreover, section Lepteranthus is represented by a single species (C. emigrantis) while section Phalolepis is represented by five species.

**CONCLUDING REMARKS**

According to our results, the success of the cross-amplification is influenced by the evolutionary distance between target and source species. Taken together, the fourteen SSR loci containing more than two alleles will provide an initial backbone for comparative genetic analyses within the genus Centaurea and therefore are selected for further works. This achievement eliminates the need to look for new loci, saving time and money. It is worthwhile highlighting that the nuclear markers provide more information than the chloroplast markers. The results reported here support previous works stating the usefulness of across-taxa amplification of microsatellite markers and should encourage other researchers to follow this approach.

Since the species included in the present work are narrow endemics and/or threatened plants, the study of its genetic diversity is an essential step to design correct strategies for its conservation management. The genetic variability information reported here is merely preliminary and a more accurate work including around 20 or 30 individuals per population is required in order to get robust conclusions regarding the genetic diversity of the species assayed.

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**Table 6.** Number of private alleles (P*A*), Shannon's Information Index (I) and observed heterozygosity (H*o*) for each species computed after the 11 polymorphic nuclear loci. SD: standard deviation.

| Species                  | P*A  | I ± SD   | H*o* ± SD |
|--------------------------|------|---------|-----------|
| C. chrysocephala         | 5    | 1.45 ± 0.13 | 0.55 ± 0.05 |
| C. emigrantis            | 5    | 1.30 ± 0.23 | 0.48 ± 0.30 |
| C. heldreichii           | 1    | 0.80 ± 0.19 | 0.30 ± 0.09 |
| C. litochorea            | 10   | 1.45 ± 0.23 | 0.45 ± 0.09 |
| C. messenicolasiana      | 6    | 1.01 ± 0.23 | 0.47 ± 0.10 |
| C. princeps              | 7    | 1.08 ± 0.20 | 0.44 ± 0.08 |

**Table 7.** Number of private alleles (P*A*), Shannon's Information Index (I) and observed heterozygosity (H*o*) for each of the 14 polymorphic nuclear loci amplified in one single species; #: parameters not computed for chloroplast loci and nuclear loci amplified. *: values computed only for C. emigrantis. SD: standard deviation.

| Loci    | P*A  | I ± SD   | H*o* ± SD |
|---------|------|---------|-----------|
| CD37    | 3    | 0.46 ± 0.17 | 0.27 ± 0.11 |
| 21CM36  | -    | 1.87 ± - | 0.37* ± - |
| 38CM22  | 3    | 0.64 ± 0.23 | 0.20 ± 0.06 |
| 42CM27  | 4    | 1.42 ± 0.22 | 0.46 ± 0.10 |
| CM17    | -    | 1.61 ± - | 0.70* ± - |
| 12B1    | 1    | 1.48 ± 0.11 | 0.56 ± 0.11 |
| 13B7    | -    | 0.93 ± - | 0.80* ± - |
| 13D10   | 4    | 1.08 ± 0.23 | 0.44 ± 0.05 |
| 17E3    | 3    | 1.37 ± 0.20 | 0.54 ± 0.08 |
| 21D9    | 7    | 1.38 ± 0.40 | 0.46 ± 0.14 |
| 28A7    | 4    | 1.43 ± 0.16 | 0.60 ± 0.09 |
| ccmp3   | 1    | 0.41 ± 0.13 | -          |
| ccmp4   | 2    | 0.09 ± 0.06 | -          |
| ccmp5   | 0    | 0.60 ± 0.11 | -          |
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