Construction of a molecular identification profile of new varieties of *Nierembergia linariaefolia* by anchored microsatellites

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Abbreviations: CTAB: Cetyltrimethylammonium bromide  
ISSR: Inter simple sequence repeat  
MIP: Molecular identification profile  
PCR: Polymerase chain reaction  
Rp: Resolving power

The objective of the present work was to establish the molecular identification profile for six new varieties of *Nierembergia linariaefolia* to incorporate the fingerprint, as complementary information to the standard registration data. Total DNA was extracted from young leaves following the protocol of the cetyltrimethylammonium bromide. Anchored microsatellites were used as molecular markers. The amplification reactions were carried out with seven primers. A total of 251 loci were detected, 98% of them were polymorphic. The average of polymorphic loci was 35 loci per primer and, 41 loci per genotype. Six out of the seven primers used discriminated all the individuals involved in the present study; consequently, it was possible to generate the molecular identification profile for the six new varieties. This result, supported together with our previous reports, indicates that the anchored microsatellites are a very useful technique for the fingerprints generation in *N. linariaefolia*.

The genus *Nierembergia*, a member of the Solanaceae family, comprises about 21 species, 15 of them are natives from Argentina (Cocucci, 1995). The distribution centre of this specie is located in the central region of the country (Zuloaga and Morrone, 1999).

Four species of the genus *Nierembergia* were incorporated to the native ornamental plants breeding program: *N. rivularis*, *N. tandilensis*, *N. calycina* and *N. linariaefolia*, which is the most advance in the breeding process.

Based on the abundant and length of the flowering period, the variability in the colour of the flowers and the easiness of propagation six new varieties of *N. linariaefolia* were selected. Four of these varieties (“Nube”, “Luna”, “Estrella” and “Cielo”) are already registered, whereas the remainders (“Bruma” and “Nieve”) are under registration process. Into this context, the molecular markers for varietal identification can be used as a tool to avoid the fraudulent multiplications as well as to preserve property rights, and they have become a complement of the traditional methods based on observable characters of the plants (Cenis, 2000).

The ISSR markers (Zietkiewicz et al. 1994) are generated...
from single-primer PCR reactions where the primer is
designed from di- or trinucleotide repeat motifs with a 5’ or
3’ anchoring sequence of one to three nucleotides (Wolfe et
al. 1998), without the requirement for prior sequence
information (McGregor et al. 2000). Its application as PCR
primer revealed high polymorphism degree (Zietkiewicz et
al. 1994), generating reliable information for DNA analysis
and with the necessary sensibility to distinguish among
individuals genetically related (Pérez de la Torre et al.
2003).

This technique was successfully applied to the study of
Astragalus oniciformi populations (Alexander et al. 2004),
Penstemon sp (Wolfe et al. 1998) and in taxonomic studies
of Vigna (Ajibade et al. 2000). In ornamental species ISSRs
have been used in Jacaranda sp (Pérez de la Torre et al.
2003; Escandón et al. 2005a), Nierembergia (Escandón et
al. 2005b), Pandorea sp (Jain et al. 1999) and
Chrysanthemum (Wolff et al. 1995). The ISSR strategy was
applied to generate fingerprints in selected clones of N.
linariaefolia, supported by the reproducibility of the
banding patterns compared to the RAPD’s ones, being the
longer primers and the higher annealing temperature the
probable causes of this difference (Nybon, 2004).

The objective of the present work was to establish a MIP to
add to the classical variety descriptors as complementary
information for cultivar registration.

MATERIALS AND METHODS

Plant material and DNA isolation

Total DNA was extracted following the CTAB procedure
according to CIMMYT, Laboratory Protocols (2005). Young
leaves of N. linariaefolia var. Nube INTA-JICA,
Luna INTA-JICA, Estrella INTA-JICA, Cielo INTA-JICA,
Nieve INTA-JICA and Bruma INTA-JICA were frozen
with liquid nitrogen and ground using mortar and pestle.
DNA quality was assessed by running 0.8% agarose gels
stained with Ethidium Bromide (1/100 v/v), using λ Hind
III as molecular weight pattern.

Polymerase chain reaction

Seven ISSR primers were used in the present study: 5’CT:
CCGGATCC(CT)n (Pérez de la Torre et al. 2003), 5’GT:
CCGGATCC(GT)n, 5’GA: CCCGGATCC(GA)n, 5’CA:
CCGGATCC(CA)n, 3’GA: (GA)nT (Blair et al. 1999),
3’AC: (AC)mG, 3’GGG: GG(TGGGG)2TG (University of
British Columbia, UBC).

PCR reactions were carried out in a final volume of 25 μl
containing 30 ng of total DNA; 0.5 U Taq polymerase
(InBio-Unicem, Tandil, Argentina); 2.5 μl of 10X reaction
buffer (InBio-Unicem, Tandil, Argentina); 0.2 mM of
dNTP’s; 0.8 μM primers and 3.0 mM MgCl2. DNA
amplifications were performed in an Eppendorf
thermocycler (Mastercycler personal) with a preliminary
step of 10 min at 94ºC, for the primers 3’AC and 3’GGG a
touch down from 47ºC to 42ºC was done, 45 sec at 47ºC,
90 sec at 72ºC and 40 sec at 94ºC, followed by 40 cycles
with the following conditions: 40 sec at 94ºC, 45 sec at
42ºC, 90 sec at 72ºC and a final 10 min extension at 72ºC.
For the other primers (5’GA; 5’CT; 5’GT, 5’CA and 3’GA)
the PCR conditions were: initial step of 10 min at 94ºC, and
40 cycles with 40 sec at 94ºC, 45 sec at 57ºC, 90 sec at
72ºC and a final 10 min extension at 72ºC.

For the analysis only the well defined and reproducible
bands were considered. Bands with the same migration
distance were considered homologous fragments,
independently of their intensity. The analysis was
performed in a visual way, with the support of the program
for calculation of molecular weight designed by Dr. Jorge
Dubcovsky (Professor of Agronomy and Extension
Department of California University, Davis, USA).

Accordingly the presence of a band was scored as “1” while
the absence of the band was scored as “0”, representing
null-alleles. Data sets derived from the respective banding
patterns were used to generate a basic data matrix for each
accession.

The power of each ISSR primer to distinguish among the
studied genotypes was evaluated by the Resolving Power
(Rp) (Prevost and Wilkinson, 1999). It is defined per
primer as: Rp= Σ Ib where Ib is the band informativeness,
that takes the values of: 1-2(x0.5-p), being p the
proportion of the six Nierembergia varieties containing the
band.
RESULTS AND DISCUSSION

Ornamental crops involve a wide range of species, most of them are almost unknown at a genetic level. Into this frame, molecular markers procedures play a relevant role in the study of the genetic diversity, in determining cultivar purity and hence leading to improve property rights protection (De Riek, 2001).

In the present work seven ISSR primers were used to generate fingerprints in selected clones of *N. linariaefolia*. The ISSR primers were described as highly conserved in most of the studied plant genomes (Blair et al. 1999). These authors evaluated the genetic diversity among 59 rice cultivars. Fernández et al. (2002) reported that sixteen barley cultivars were distinguished applying this technique. Jain et al. (1999) evaluated the genetic diversity and generated genome fingerprinting of genus *Pandorea*. In the same way, Pérez de la Torre et al. (2003) and Escandón et al. 2005a discriminated among 6 and 21 accessions of *J. mimosifolia* respectively.

Figure 1 show, as an example, the amplification profile obtained with the 5'CA, 3'AC and 3'GGG primers. The analysis of bands per primer in the present gel, shows, for the primer 5'CA polymorphic loci in: 1243, 1082, 1011, 878, 857, 791, 744, 709, 683, 658, 615, 584, 577, 549, 531, 495, 457, 416, 385, 371 and 361 bp. For the primer 3'AC, the bands that revealed polymorphic loci were: 1169, 1049, 1005, 980, 951, 933, 862, 846, 801, 787, 754, 735, 687, 630, 545, 528, 501, 491, 445 and 374 bp. Finally, the primer 3'GGG shows polymorphic loci at: 999, 911, 889, 796, 782, 642 and 498 bp.

The generated MIP for each variety of *N. linariaefolia* is shown in Table 1.

Table 2 summarized the data obtained: the number of total loci (TL), the number of polymorphic loci (PL), the percentage of polymorphic loci (%P), the number of different genotypes identified (IG) and the Rp of each primer. The seven primers used detected a total of 251 loci, 98% of them were polymorphic, and an average of 41 loci per genotype and 35 loci per primer. The percentage of polymorphic loci ranged from 93.1% for 3'GGG to 100% for 5'CA (Table 2).

Prevost and Wilkinson (1999) reported the Rp as the capacity of a given primer to discriminate among different genotypes. As can be seen in Table 2, with primers that show a Rp value equal or higher than 4.83 it was possible to discriminate among the six *Nierembergia* varieties.

Primers 5'CA, 5'GT, 5'CT and 3'GA were tested in a previous study with other very related genotypes (Escandón et al. 2005b). The Rp values obtained in the mentioned report were different of the corresponding values obtained here, being the most dramatic case for primer 5'CA, that gave a Rp value of 13.67 in the present study in contrast with a value of 5.5 obtained of the previous report (Escandón et al. 2005b). This fact confirms that the power of a primer to discriminate among different genotypes must be circumscribed to the set of individuals involved in each study.

In this report 246 new traits are presented. These traits, together with the morphological ones are excellent tools for the differentiation of *Nierembergia* varieties.
CONCLUDING REMARKS

A MIP was obtained for each of the six new varieties of *N. linariaefolia* presented in this paper.

All primers, with exception of 5'GA that discriminated among 4 varieties only, were able to distinguish among the genotypes analyzed in this study.

The anchored microsatellites showed to be an economic, fast, and simple technique, besides reproducible and reliable for the generation of fingerprints and the establishment of genetic relations in *N. linariaefolia*.

### REFERENCES


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