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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

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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).

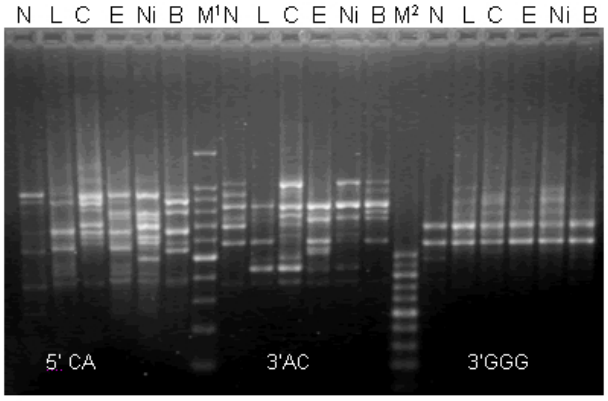

Figure 1. Amplification products obtained from six varieties of *N. linariaefolia* using primers 5'CA, 3'AC and 3'GGG. N: var. Nube, L: var. Luna, C: var. Cielo, Ni: var. Nieve, B: var. Bruma, M^1:100 bp ladder, M^2: 50 bp ladder.

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 *Chrysantemum* (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% agarosa 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: $CCGGATCC(CT)_9$ (Pérez de la Torre et al. 2003), 5'GT: $CCCGGATCC(GT)_9$, 5'GA: $CCCGGATCC(GA)_9$, 5'CA: $CCCGGATCC(CA)_9$, 3'GA: $(GA)_9T$ (Blair et al. 1999), 3'AC: $(AC)_8G$, 3'GGG: $GGG(TGGGG)_2TG$ (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 $MgCl_2$. 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.

10 µl of PCR products were analyzed on 2.5% agarose gels in TAE buffer running at 60 V for 240 min. Gels were stained with Ethidium Bromide (5/100 v/v). The bands obtained were sized (in base pairs) by comparison with standard marker (100 bp and 50 bp ladder, PB-L, UNQ, Quilmes, Argentina).

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: $Rp= \Sigma Ib$ where Ib is the band informativeness, that takes the values of: 1-(2x[0.5-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).

Table 1. Molecular identification profile of each *N. linariaefolia* variety obtained with all primers used in the present work. * indicates the presence of a *locus* in the gel.

5'CA	N	L	C	E	Ni	B
1243			*			
1082			*		*	
1011		*				*
951	*	*	*	*	*	*
878	*	*		*		*
857			*			
816			*			
791	*			*	*	*
744				*	*	
709		*	*			
683					*	
658	*	*	*	*		*
615		*	*	*	*	
584	*				*	*
577			*	*		
549		*		*	*	
531	*					*
495					*	
457		*		*		
416		*		*	*	
385				*	*	*
371		*	*			
361	*					

5'GT	N	L	C	E	Ni	B
979					*	
895	*	*		*		
879					*	*
795		*		*		
719					*	*
711			*			
686	*					
666		*		*	*	*
647		*				
606		*				
595	*		*			
568					*	
528		*		*		*
512			*		*	
497	*					*
482		*	*		*	
447						*
418	*		*		*	*
384				*	*	*
355				*	*	
334	*	*				*

3'AC	N	L	C	E	Ni	B
1169					*	*
1049	*		*			
1005					*	*
980			*			
951	*					
933			*			
862					*	*
846	*	*	*	*		
801						*
787		*		*		
754	*		*		*	*
735		*		*		
687	*		*			
630			*		*	
596	*	*	*	*	*	*
545				*		
528				*		
501			*			
491		*				
445		*	*	*	*	
374	*	*	*	*	*	

3'GA	N	L	C	E	Ni	B
781						*
754					*	
686	*	*	*	*		*
585						*
509		*				
494				*		
491	*					
476		*		*	*	*
434				*	*	*
412	*	*		*	*	*
384	*	*	*	*	*	*
341			*	*	*	*
309			*			

5'CT	N	L	C	E	Ni	B
962				*		
754				*		
662		*		*		
610				*	*	
554			*			*
541	*	*		*	*	
500			*			*
459		*	*	*	*	*
394			*			
352			*			
318			*	*	*	*

3'GGG	N	L	C	E	Ni	B
999		*		*	*	
911			*		*	*
889		*				
796			*	*	*	*
782		*				
709	*	*	*	*	*	*
642		*	*	*	*	
584	*	*	*	*	*	*
498	*					

5'GA	N	L	C	E	Ni	B
1208			*			
724		*		*		*
542	*	*		*		*
509			*			
502					*	
434		*		*		

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, 816, 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'CT, 5'GT y 5'GA. Rp ranged from 4.33 for 5'GA to 13.67 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.

Together with the stability and the uniformity, to be different is a necessary requisite for a variety registration; the ISSR have shown to be an adequate tool for the differentiation of varieties being a true fingerprint of each genotype. 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*.

Table 2. Summary of the results obtained in the construction of a molecular identification profile of new varieties of *N. linariaefolia*. TL: number of total *loci*, LP: polymorphic *loci*, %P: percentage of polymorphic *loci*, IG: number of genotypes identified per primer and Rp: Resolving power.

Primer	TL	PL	%P	IG	Rp
5'CT	25	25	100,00	6	5,67
5'GT	44	44	100,00	6	12,67
3'GA	34	33	97,06	6	5,67
5'CA	57	56	98,25	6	13,67
3'AC	47	46	97,87	6	7,83
3'GGG	29	27	93,10	6	4,83
5'GA	15	15	100,00	4	4,33
TOTAL	251	246	98,01		

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