Genetic diversity of blue-flowered *Scilla* species as determined by random amplified polymorphic DNA

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The phylogenetic relationships among three *Scilla* species (*S. natalensis*, *S. kraussii* and *S. dracomontana*) and the man-made hybrid (*S. natalensis* X *S. kraussii*) were studied using random amplified polymorphic DNA (RAPD) markers. Distinct RAPD patterns were obtained for the three *Scilla* species and the hybrid with seven different random oligonucleotide primers. The results suggest that the three blue-flowered *Scilla* taxa are distinct species. Analyses of the RAPD products of the hybrid and the parent species revealed shared inherited polymorphism. The results indicate that RAPD markers can be applied successfully for the identification of economically important *Scilla* species.

Introduction

The genus *Scilla*, which can be either narrowly or broadly defined, is placed in the family Hyacinthaceae (Dahlgren *et al.* 1985). The exact number of species of *Scilla* represented in the southern African flora is uncertain. Baker (1897) recognised 56 species (in a broadly defined genus), whereas Obermeyer in Arnold and De Wet (1993) recognised four (in a narrowly defined genus), based largely upon Jessop's taxonomic revision of *Scilla*, *Schizocarpus* and *Ledebouria* (Jessop 1970). One of these is *Scilla natalensis* Planch. Reduced to synonymy in this species are *S. kraussii* Baker and *S. dracomontana* Hilliard & B.L.Burtt.

The opinions expressed by Jessop (1970) are not shared by all taxonomists. Although *S. natalensis* (*sensu stricto*), *S. kraussii* and *S. dracomontana* are all blue-flowered, they differ from one another in habit and vegetative morphology (Figure 1 and Table 1). Griffioen and Edwards (1994) having analysed gross morphology, anatomy, micro-morphology of seed, pollen and leaf epidermal pattern, palynology and cytology concluded that the three blue-flowered taxa are distinct, an opinion followed by Pooley (1998).

Molecular markers which reveal extensive polymorphism at the DNA level are powerful tools in the identification of plant species. One of the commonly used molecular markers is random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990). RAPDs has been used to confirm somatic hybrids (Xu *et al.* 1993, Takemori *et al.* 1994), genetic properties of micropropagated plantlets (Rani *et al.* 1995), geographic variation of plants (Nakai *et al.* 1996, Rajaseger *et al.* 1999) and to evaluate the genetic relationship between plant species (Rath *et al.* 1998, Sastad *et al.* 1999).

In South Africa the bulbs of *S. natalensis* are used extensively as a traditional medicine. Homo *iso*-flavanones have been isolated from the bulbs of all three blue-flowered *Scilla* species (Crouch *et al.* 1999). Van Wyk *et al.* (1997) suggested that the wound-healing, anti-septic and anti-inflammatory properties of these bulbs are possibly due to the presence of these homo *iso*-flavanones. McCartan and Van Staden (1998) developed a method for the micropropagation of the large *S. natalensis*, which could facilitate conservation by cultivation, alleviating pressure on natural resources. Since an increasing amount of research is focusing on the traditional uses and benefits of these plants as well as their horticultural potential, it is necessary to resolve the taxonomic uncertainty surrounding the number of infrageneric taxa in genus *Scilla s. str.* The aim of this study was to obtain conclusive data concerning the genetic relationship of the three blue-flowered species and an artificial hybrid (created by Mr R Roth) using RAPD techniques.

Material and Methods

Samples from fully expanded leaves of *Scilla natalensis*, *S. kraussii*, and *S. dracomontana*, originally collected from their natural habitats in KwaZulu-Natal and now growing in the University of Natal Botanical Gardens, and an artificial hybrid, *S. natalensis* X *S. kraussii*, were collected in triplicate. Leaves were washed, surface-sterilised and frozen with liquid nitrogen and then stored at -70°C for subsequent genomic DNA isolation.

Genomic DNA was isolated according to Pich and...
Table 1: Salient characteristics of the three blue-flowered Scilla species subjected to the RAPD analysis

| Plant                   | Height | Habitat                           | Bulb                                      | Leaves                                | Inflorescence                      |
|-------------------------|--------|-----------------------------------|-------------------------------------------|---------------------------------------|------------------------------------|
| S. natalensis Planch.   | Up to 1m| Damp grassland, cliffs and rocky slopes, coast to 2000m | Approximately 300mm in diameter, half above ground, papery, purplish brown | Erect, approximately 500mm long, hairless or velvety, produced after flowers | 300 X 100mm; flowers pale to deep purplish blue (September – October) |
| S. kraussii Baker       | Up to 250mm| Rocky hillsides, below 1000m| Roundish, approximately 50mm in diameter | Spreading, 50–75mm long, firm, strongly ribbed, softly hairy, green above, purplish beneath after flowers | 45–150mm; flowers approximately 10mm in diameter (January) |
| S. dracomontana        | Up to 110mm| Cliffs, rock platforms, 1675–2100m | Small, dwarf bulbs, 10–15mm in diameter | Flat on the ground                      | 25–45mm; flowers blue (September – November) |

Schubert (1993). Leaf material (100mg), was quickly frozen in liquid nitrogen, powdered in a mortar and pestle, and 600μl extraction buffer (500mM NaCl, 50mM Tris HCl pH 8.0; 50mM EDTA; 1% 2-mercaptoethanol) added. The mixture was thawed on ice and 260μl ice cold PVP stock solution added, whereasafter 17.6mg solid SDS was added. The mixture was incubated for 10min at 65°C. Eighty-six μl 5M potassium acetate were added followed by 30min incubation on ice and centrifugation (10 000g) for 10min. The supernatant was transferred into a new test-tube and mixed with 0.6 volume of isopropanol and incubated on ice for 10min. After another centrifugation (10 000g) for 10min the supernatant was discarded and the pellet dried under vacuum. DNA was dissolved in 500μl TE buffer and extracted once with 1 volume of phenol/chloroform/iso-amylalcohol (25:24:1). After centrifugation (1 000g for 10min at 4°C), the aqueous phase was transferred and DNA was precipitated in 1 volume isopropanol. The DNA pellet was washed in 70% ethanol, dried under vacuum and dissolved in TE buffer and the DNA further purified by treating with RNase (10μg/ml, 37°C, 30min) and proteinase K (12μg/ml, 37°C, 30min), respectively. This was followed by a phenol/chloroform extraction step. The DNA was precipitated by adding 2.5 volumes of pre-cooled absolute ethanol and centrifuged (10 000g) for 10min at 4°C. The pellet was dissolved in 50μl TE buffer and the DNA was quantified using a CARY 50 CONC UV-Visible Spectrophotometer. DNA was diluted to 200ngμl−1 for RAPD analyses.

RAPD analyses were carried out with varying concentrations of MgCl2 (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0mM) and DNA template (100, 150, 200, 250, 300, 400ng) to optimise the PCR conditions. Taq (2.5 units), dNTPs (0.4mM) and primers (1μM) were used.

For initial primer screening, a reaction mixture (25μl) contained: 1 x PCR buffer (Roche, Germany), 1.5mM MgCl2, 0.4mM of each dNTP (Roche, Germany), 1μM primer, 2.5 units of Taq (Roche, Germany) and 1μl of 200ngμl−1 template DNA. The negative control mixture consisted of all reagents, except the DNA template. Each reaction mixture was overlaid with 30μl of mineral oil to prevent evaporation. Amplification reactions were duplicated to ensure reproducibility. The amplification was performed in a Hybaid Thermal Reactor (Hybaid Limited, UK) programmed for pre-denaturation (95°C, 2min), followed by 45 cycles which consisted of denaturation (94°C, 10sec), annealing 36°C, 20sec and extension (72°C, 2min) with a final extension (72°C, 10min).

Twenty-four random primers, each consisting of ten nucleotides (OPA, OPB), were purchased from Operon Technologies, Inc. (Alameda, CA, USA). Seven primers that gave reproducible results in three independent DNA extractions were then chosen for further analyses. Their sequences (5’ to 3’) are as follows: OPB8: GTGACGTAG; OPA14: TCTGTGTCTG; OPA16: AGCCAGC; OPA18: AGGTACC; OPA19: CAAAGTCC; OPB7: GTGAGCAG. The amplification products together with a size DNA marker (100bp DNA ladder, Pharmacia) were separated on 1.6% (w/v) agarose gels at 70 volts for 3h. The gels were stained with 0.5μg/ml ethidium bromide and photographed under UV light. The RAPD bands were scored as discrete variables, using 1 to indicate presence and 0 to indicate absence of a band. A pairwise difference matrix between genotypes was computed using the simple matching coefficient. A phenogram was generated using the unweighted pair-group method with arithmetical averages as described by Sneath and Sokal (1973).

Results and Discussion

High quality DNA was obtained from all three Scilla species and the hybrid. In the PCR reactions best results were obtained with concentrations of 200ng DNA template and 1.5mM MgCl2. Initially 24 random primers were used in the screening process. Seven primers generated reproducible RAPD profiles (i.e. each primer generated at least two identical RAPD profiles). In all, the seven primers generated 266 reproducible bands ranging from 150 to 2 000 bp (Table 2). Representative RAPD profiles generated with primers OPB8, OPA14, OPA17 and OPB7 are shown in Figure 2.

Despite exhibiting polymorphisms, S. natalensis, S. kraussii and S. dracomontana yielded several common bands. However, some very distinct bands were also observed, providing a useful tool to distinguish among the Scilla species and the hybrid. S. dracomontana could be differen-
Figure 1: Appearance of the blue-flowered Scilla species investigated in this study. (A and B) Scilla natalensis; (C) S. kraussii; (D) S. dracomontana; (E) Artificial hybrid of S. natalensis X kraussii
Table 2: Total number of RAPD bands generated for three Scilla species and an artificial hybrid by the seven random primers used

| Primer | S. natalensis | S. kraussii | S. natalensis X kraussii | S. dracomontana | Total |
|--------|---------------|-------------|--------------------------|-----------------|-------|
| OPA8   | 8             | 6           | 6                        | 6               | 26    |
| OPA14  | 6             | 5           | 7                        | 9               | 27    |
| OPA16  | 11            | 12          | 10                       | 16              | 49    |
| OPA18  | 5             | 10          | 6                        | 3               | 24    |
| OPA19  | 14            | 12          | 12                       | 11              | 49    |
| OPB1   | 19            | 17          | 17                       | 14              | 67    |
| OPB7   | 3             | 8           | 5                        | 9               | 24    |
| Total  | 66            | 70          | 62                       | 68              | 266   |

Table 3: Various RAPD bands* generated for the Scilla species and an artificial hybrid by the random primer OPA8

| Markers (bp) | S. natalensis | S. kraussii | S. natalensis X kraussii | S. dracomontana |
|--------------|---------------|-------------|--------------------------|-----------------|
| 1600         | 1             | 1           | 0                        | 0               |
| 1400         | 1             | 0           | 0                        | 0               |
| 1100         | 0             | 0           | 0                        | 1               |
| 1000         | 0             | 0           | 0                        | 1               |
| 900          | 1             | 1           | 1                        | 1               |
| 800          | 1             | 1           | 1                        | 0               |
| 600          | 0             | 0           | 0                        | 1               |
| 580          | 1             | 0           | 0                        | 0               |
| 540          | 0             | 1           | 0                        | 0               |
| 520          | 0             | 0           | 0                        | 1               |
| 500          | 0             | 0           | 0                        | 1               |
| 480          | 1             | 0           | 0                        | 0               |
| 450          | 0             | 1           | 0                        | 1               |
| 420          | 0             | 0           | 0                        | 1               |
| 300          | 1             | 0           | 1                        | 1               |
| 280          | 0             | 0           | 0                        | 1               |
| 200          | 1             | 1           | 1                        | 0               |

* Bands scored according to presence (1) or absence (0)

ticated clearly from S. natalensis and S. kraussii on the basis of the absence of specific DNA bands (200 and 800bp) and the presence of the 280, 500, 600, 1000 and 1100bp using random primer OPA8 (Table 3).

Data obtained from all seven primers, used for all plant material analysed, were used for cluster analysis, and a phenogram constructed (Figure 3). Analysis of the RAPD profiles indicated that the blue-flowered species can be divided into two broad groups at the level of 0.53. The first group includes S. natalensis and S. kraussii at the level of 0.69. S. natalensis is a tall form and S. kraussii a middle-sized form (Table 1). The bands at 200, 800, 900 and 1 600bp were common to the two species. They could be distinguished from each other by the presence of the 300, 480, 580 and 1 400bp in S. natalensis in the RAPD profiles generated by primer OPA8 (Table 3). The second group includes S. dracomontana only which is a very small form. These results support the notion that the three species are distinct taxa (Griffioen and Edwards 1994, Pooley 1998).

Cluster analysis of S. natalensis and S. kraussii with the hybrid (S. natalensis X kraussii) indicated that the hybrid is closely (0.76) associated with S. natalensis, and less closely with S. kraussii (Figure 3). RAPD profiles of the two parents with the seven random primers tested were distinct from those of the hybrid. One set of representative profiles

Figure 2: RAPD profiles of S. natalensis (lanes 2, 6, 10, 14), S. kraussii (lanes 3, 7, 11, 15), S. natalensis X kraussii hybrid (lanes 4, 8, 12, 16) S. dracomontana (lanes 5, 9, 13, 17) generated from primers OPA8, OPA14, OPA18 and OPB7 after fractionation by agarose (1.6%) gel electrophoresis. Lane one represents the molecular markers.
Figure 3: Phenogram generated for blue-flowered Scilla species and an artificial hybrid indicating genetic relationships and percentage similarity. All RAPD bands (266 in total) scored from seven random primers were used for the calculations.

generated by primer OPB7 is shown in Figure 2. Scilla natantensis, the hybrid (S. natantensis X S. kraussii) and S. kraussii exhibited one common band (1300bp), which might explain the success of hybridisation between the two species. The bands at 200bp and 500bp were specific for the hybrid and S. natantensis only. Similarly, the band at 800bp was specific for S. kraussii and the hybrid. Our observations indicate that S. natantensis is the female parent of the hybrid.

We are acutely aware of the limitations involved in basing taxonomic conclusions on a small sample size of the taxa concerned, as is the case in this study. It is nevertheless hoped that this data, combined with existing macromorphological and ecological evidence, will contribute towards the acceptance of the three blue-flowered Scilla taxa as three distinct species.

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