Intraspecific diversity in *Sinningia speciosa* (Gesneriaceae: Sinningieae), and possible origins of the cultivated florist’s gloxinia

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Received: 21 June 2012; Revised: 9 October 2012; Accepted: 13 October 2012; Published: 24 October 2012

Citation details: Zaitlin D. 2012. Intraspecific diversity in *Sinningia speciosa* (Gesneriaceae: Sinningieae), and possible origins of the cultivated florist’s gloxinia. AoB PLANTS 2012: pls039; doi:10.1093/aobpla/pls039

Abstract

Background and aims The florist’s gloxinia is a familiar houseplant in the Gesneriaceae, the botanical family that includes the African violet (*Saintpaulia*) and other ornamental species. The gloxinia’s wild progenitor is *Sinningia speciosa* (Lodd.) Hiern, a Brazilian endemic. Although it has been cultivated for almost 200 years, little is known about the genetic diversity in *S. speciosa*, how the wild populations relate to one another or even where the cultivated forms originated. Using available wild collections, preliminary phenetic and phylogenetic investigations were conducted to elucidate the interspecific relationships within *S. speciosa* and to infer the origins of the cultivars.

Methodology Amplified fragment length polymorphism (AFLP) analysis was applied to 24 accessions of *S. speciosa* (17 wild collections, seven cultivars) and one accession each of *Sinningia guttata* and *Sinningia macrophylla*. A maximum likelihood (ML) tree was also calculated from an alignment of the nuclear ribosomal internal transcribed spacer sequence from the same 26 accessions.

Principal results Dice/UPGMA and principal coordinates analysis of the AFLP data partitioned *S. speciosa* into several distinct clusters, one of which included *S. macrophylla*. All cultivated ‘gloxinias’ grouped together in a major cluster with plants from Rio de Janeiro. The AFLP results were compared with a phylogenetic analysis of the ribosomal spacer region, which was informative in *S. speciosa*. The ML tree generally supported the AFLP results, although several clades lacked strong statistical support.

Conclusions Independent analyses of two different data sets show that *S. speciosa* is a diverse species comprised of several lineages. Genetic distance estimates calculated from the AFLP data were positively correlated with geographic distances between populations, indicating that reproductive isolation could be driving speciation in this taxon. Molecular markers are under development for population genetic studies in *S. speciosa*, which will make it possible to define evolutionarily significant units for purposes of conservation.

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AoB PLANTS 2012: pls039; doi:10.1093/aobpla/pls039, available online at www.aobplants.oxfordjournals.org © The Authors 2012
Introduction

Sinningia speciosa is a herbaceous, tuber-forming perennial native to south-eastern Brazil, and is one of the ~70 species in the genus Sinningia (Gesneriaceae, tribe Sinningieae) (Chautems et al. 2010). Sinningia speciosa was originally introduced into cultivation in Great Britain in 1815 (Don 1838; Harrison 1847). The first known collection was made by Allan Cunningham (1791–1839) and his partner James Bowie (1789–1869), both of whom were employed as Botanical Collectors in Brazil by the Royal Botanic Gardens, Kew, from 1814 to 1816 (Heward 1842; Britten and Boulger 1893; Smith 1911). Joachim Conrad Loddiges (1738–1826), the owner of a large and prestigious nursery in the village of Hackney (now a borough of London), introduced S. speciosa to the public with an illustration in the first issue of The Botanical Cabinet (Loddiges 1817), published by his son George from 1817 to 1833 (Fig. 1). Loddiges named the new plant Gloxinia speciosa, placing it in Gloxinia L’Héré-tier, an existing genus of rhizomatous perennial herbs in the Gesneriaceae from Central and South America. The flowers of the type species, Gloxinia perennis, are outwardly similar to those of S. speciosa, and Loddiges’ understandable error accounts for the common name ‘gloxinia’ came into general use for this species. Improved cultivars have been grown as ornamental houseplants and in greenhouses for over 160 years in Great Britain, continental Europe and the United States, and are still grown to this day. Wild forms of S. speciosa all have nodding, bilaterally symmetrical flowers that are lavender or purple (rarely white or pink) in colour. Cultivated varieties, however, have fully erect, radially symmetrical (peloric) flowers in colours ranging from white to purple and red, and often with unusual corolla patterning (Fig. 2). Of the many hundreds of cultivars named and sold in the 19th and early 20th centuries, few have survived to the present day; examples of some that are still available are ‘Emperor Fredrick’, ‘Emperor William’ (aka ‘Kaiser Wilhelm’), ‘Defiance’ and ‘Blanche de Meru’.

The distribution of S. speciosa is restricted to the Atlantic coastal forests (Mata Atlântica) of south-eastern Brazil (Araujo and Chautems 2010), an area recognized as one of 34 world biodiversity hotspots because of its high level of species richness and endemism (Myers et al. 2000; Mittermeier et al. 2004). The geographical range of S. speciosa encompasses ~4 x 10^5 km² in Espírito Santo, Minas Gerais and Rio de Janeiro states, and populations may also exist in São Paulo, Paraná and Santa Catarina states (A. Chautems, Conservatoire et Jardin Botaniques de la Ville de Genève, personal communication). Human activities such as agriculture (cattle ranching; sugarcane, eucalyptus and coffee plantations), mining, and expansion of the major cities of Rio de Janeiro and São Paulo have greatly reduced the size of the southern coastal forests, threatening the genetic diversity of the plant and animal species that live there. Despite this loss of habitat, many natural populations of S. speciosa survive, and the species as a whole has retained considerable morphological diversity (Zaitlin and Pierce 2010). Variation is readily observed in the leaves (size, shape, colour, indument), flowers (size, shape, number per axil), corolla (colour, shape, patterning), petal lobes (size, shape), calyx (size, shape) and stem (colour, internode length) [see Additional Information]. Past taxonomic treatments of S. speciosa have left us with a list of discarded synonyms for this diverse species encompassing 39 taxa in seven genera that
included Ligeria, Orthanthe, Orobanche (Skog and Boggan 2007) and Martynia (Loiseleur-Deslongchamps 1820) in addition to Gloxinia and Sinningia.

The objective of the research presented here was to determine whether amplified fragment length polymorphisms (AFLPs) are informative in *S. speciosa*, and to test the hypothesis that they can be used for investigating genetic diversity within a group of wild and cultivated accessions. While the main goal of this preliminary study was to define the intraspecific relationships between plants collected from natural populations, seven domesticated cultivars were also included to shed some light on the geographical origins of the cultivated florist’s gloxinia. Amplified fragment length polymorphism is a powerful and robust molecular marker technology that requires no prior knowledge of genome sequence, and is therefore potentially useful in non-model and genetically uncharacterized...
organisms. Amplified fragment length polymorphisms have been widely applied in studies of plant genetic diversity and evolution (Sudupak et al. 2004; Spooner et al. 2005; Zerega et al. 2005; Allen et al. 2008; Azizi et al. 2009; Garrido et al. 2012), genotyping and cultivar identification in ornamental and horticultural crops (Chao et al. 2005; Parks et al. 2006; Krichen et al. 2008; Wu et al. 2011), and as an aid in resolving species-level phylogenies when DNA sequence diversity is limited (Beardsley et al. 2003; Despres et al. 2003; Pelser et al. 2003; Meredda et al. 2008). In the present study, an analysis of fluorescent end-labelled AFLP fragments amplified from genomic DNA was used to infer genetic relationships within a group of *S. speciosa* accessions. Included were 15 wild collections of *S. speciosa* of known origin, two wild-type collections of unknown origin, seven ‘gloxinia’ cultivars and two related species (*Sinningia guttata* and *Sinningia macrophylla*) as potential outgroups. The 26 plant accessions were clustered using phenetic (Dice/UPGMA) and principal coordinates analyses (PCoA) of the AFLP data. These results were compared with a phylogenetic analysis of the nuclear ribosomal internal transcribed spacer (nITS) region amplified and sequenced from the same 26 *Sinningia* taxa. Taken together, the two independent analyses show that *S. speciosa* is a highly diverse taxon that may include *S. macrophylla*, and that the *S. speciosa* cultivars appear to be most closely affiliated with plants collected from populations in or near the city of Rio de Janeiro.

**Methods**

**Plants and DNA**

The majority of plants used in this study were grown from seeds, with several exceptions as detailed below. Eight of the wild-collected *S. speciosa* accessions, as well as the cultivars ‘Guatapara’ and ‘Dona Lourdes’, were obtained as seeds from M. Peixoto (Mogi das Cruzes, SP, Brazil) and are described in Zaitlin and Pierce (2010). The wild *S. speciosa* collections ‘Massaguassu’ (from near a beach of that name in the municipality of Caraguatatuba in SP state) and ‘CM’ (unknown origin, possibly from Rio de Janeiro), and the cultivar ‘Purple’ were also obtained from M. Peixoto. Wild-type *S. speciosa* accessions AC1503, ‘Chiltern Seeds’ and ‘Regina’, and the peloric cultivar ‘Red with Spots’ were purchased from the seed fund of the Gesneriad Society (www.gesneriadsociety.org). Seeds of the peloric white-flowered cultivar ‘Ken’s White’ were obtained from the Gesneriad Hybridizer’s Association. ‘Avenida Niemeyer’ was the kind gift of Wallace Wells of New York, NY; the original collection was made in August 1975 by Charles Marden Fitch on a rocky slope between the Sheraton
Rio Hotel and the Hotel Nacional in Rio de Janeiro on the coastal road of this name. Sinningia speciosa ‘São Conrado’ was collected in the same general area in 1999, several kilometres to the west, by Mr Tsuch Yang Chen of New York City, and was the gift of Dr William Price of Vancouver, BC, Canada. ‘Kaiser Wilhelm’, a classic ‘gloxinia’ cultivar with peloric purple and white flowers, was obtained as a tuber from a commercial source. DNA of JFS4512, a peloric cultivar, was kindly provided by Dr James Smith of Boise State University (Smith et al. 2004). Sinningia seeds were routinely germinated in 10 cm pots containing Pro-Mix BX (Premier Horticulture Inc., Quakertown, PA, USA), and the seedlings were grown under fluorescent lighting (80 μmol m⁻² s⁻¹) at 25 °C until they were large enough to sample for DNA extraction.

Total plant DNA was extracted from small samples of leaf tissue (pooled from several plants of each accession) using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). DNA concentration was determined by absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

AFLP methods

Amplified fragment length polymorphism manipulations were performed essentially as described by Vos et al. (1995), using a modification that allowed for fluorescent detection of the amplified fragments. An AFLP Core Reagent Kit (catalogue #10482-016) was purchased from Invitrogen (Carlsbad, CA, USA). Fluorescent Eco + 3 primers were 5′-end labelled with the WellRed D4 dye (Beckman Coulter, Fullerton, CA, USA), and were synthesized by Integrated DNA Technologies, Inc. (IDT: Coralville, IA, USA). Unlabelled primers were also purchased from IDT. Genomic DNA samples (50–100 ng) were digested to completion with EcoRI and MseI at 37 °C in a final volume of 25 μL. Following ligation of the EcoRI- and MseI-specific adaptor sequences for 15 h at 20 °C, the reactions were diluted 10-fold into 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0). Pre-selective amplifications (PSA) and selective amplifications (SA) were performed exactly as described by Zhang et al. (2012). A C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) was used for all AFLP amplifications. Selective amplification reactions were diluted 30-fold into sample loading solution (Beckman Coulter) containing a 1/100 dilution of DNA Size Standard-600 (Beckman Coulter) for analysis. DNA fragments were separated by capillary electrophoresis on an automated DNA sequencing instrument (Beckman Coulter CEQ8000 Genetic Analysis System) using the Frag-4 method (capillary temperature = 50 °C, denaturation 90 °C for 2 min, sample injection 30 s at 2 kV, electrophoretic separation 65 min at 4.8 kV). Sequences of the AFLP primers used are given in Table 1.

DNA fragment peak sizes in the D2 channel were calculated against the standards in the D1 channel using the software supplied with the CEQ8000 instrument. The quartic equation gave the best approximation of a linear relationship between peak sizes (in nucleotides) vs. migration time with the 600 standard. Data peaks were machine scored (1 = present, 0 = absent) using the ‘New AFLP Analysis’ module (parameter settings: 10 % slope threshold, 10 % relative peak height threshold, 95 % confidence level, bin width = 1 bp, PA ver. 1 dye mobility calibration) and the data exported to Microsoft Excel. Every AFLP chromatogram was examined for the presence of unscored peaks at a minimum height threshold of 5000 fluorescence units. Data entries from poorly resolved and miscalled peaks were removed from the spreadsheets. Peaks well outside the standard size range (<60 and >640 bases) were also removed. Similarity matrices based on the Dice coefficient (Dice 1945) were calculated from the binary AFLP data using the SIMQUAL module in the NTSYSpc 2.2 software package (Rohlf 2009), and clustering was performed using the UPGMA (unweighted pair-group method with arithmetic mean) algorithm in the SAHN module. Goodness of fit of the UPGMA dendrogram to the Dice similarity matrix was assessed using the COPH and MXTCOMP modules in NTSYSpc. Bootstrapping (BS) was

Table 1: DNA sequences of oligonucleotide primers used for AFLP analysis and amplification and sequencing of the nrITS region in Sinningia. Amplified fragment length polymorphism primers E32 (Eco + AAC) and E33 (Eco + AAG) were labelled at the 5′ termini with WellRed dye D2.

| Primer name | DNA sequence (5′ → 3′) |
|-------------|------------------------|
| E01         | AGACTGGTCTGACAATTCA    |
| M02         | GATGATTCCTGAGTAAC      |
| E32         | GACTGCTGATCAAATTCAAC   |
| E33         | GACTCGTGACAAATCCAAG    |
| M48         | GATGAGTCTGAGTAACAC     |
| M49         | GATGAGTCTGAGTAACAG     |
| M50         | GATGAGTCTGAGTAACAT     |
| ITS2Gm      | TGAGGCCCAGCCAGACGT     |
| ITS3P       | GCATGATAGAAAGACGTC     |
| ITS5seq2    | CAAAGTTTCCTGAGGGAACCTG |
| ITS6        | GCGAGAAGTCCATTACCAAC   |
| ITS8m       | GACGCTTCTCCAGACTACA    |
also performed with NTSYSpc using the program modules RESAMPLE to generate 1000 resampled files of the AFLP data, SIMQUAL to calculate the similarity matrices and CONSENS (with MAJRLJ selected) to compare the trees and calculate cluster frequencies. Principal coordinates analysis was performed on the AFLP data (imported in spreadsheet format) using the statistical software package MVSP 3.2 (Kovach Computing Services, Anglesey, UK) with the following program settings: data matrix transformed, Euclidean distances computed, eigenanalysis tolerance set to $10^{-7}$. The resulting graphs were exported as enhanced metadefiles, and text labels were added with the program Metafile Companion (Companion Software, Sunderland, MA, USA).

Genetic distances (GDs) were calculated with NTSYSpc for the AFLP data (GD = 1 – Dice’s coefficient) and MEGA 5.03 (Tamura et al. 2011) for the nrITS DNA sequence data (Tamura–Nei substitution model), respectively. Geographic distances were calculated with the GPS Visualizer tool (http://www.gpsvisualizer.com/calculators) based on GPS coordinates for Brazilian cities and towns kindly provided by A. Chautems. Sinningia speciosa ‘Jurapé’ was named for the mountain in Santa Catarina state where it was allegedly collected, and coordinates for the nearest town (Joinville) are S26° 18′ 16.2″, W48° 50′ 55.248″ (–26.3045, –48.84868). Serra da Vista is a mountain ~20 km south of the town of Cardoso Moreira in Rio de Janeiro state. Coordinates used for this collection were S21° 39′ 55.116″, W41° 36′ 59.4″ (–21.66531, –41.6165). Mantel tests were performed with IBDWS (Isolation-by-Distance Web Service v.3.23; Jensen et al. 2005) to determine whether the GD matrix was significantly correlated with the geographic distance matrix. Ten thousand randomizations were used.

Nuclear ribosomal internal transcribed spacer region amplification and DNA sequencing

The internal transcribed spacer region of the 18S–26S nuclear rDNA repeat (nrITS) was amplified from Sinningia genomic DNA (10–20 ng) using primers ITS6 (this study; see Table 1 for primer sequences) and ITS5G, which direct DNA strand synthesis from the fragment ends, and ITS2Gm and ITS3P, which are complementary to the ends of the 5.8S rDNA sequence, but are of opposite polarities (Möller and Cronk 1997). The nrITS regions from all Sinningia accessions reported here were sequenced at least twice, and the sequence tracings were carefully examined to resolve any possible conflicts or errors. The nrITS region from the cultivar ‘Kaiser Wilhelm’ proved to be polymorphic; the amplified fragment was therefore cloned into pGEM-T (Promega, Madison, WI, USA), and the inserts in several recombinant plasmids were sequenced with the same four primers.

Alignment of the nrITS region was performed manually using the program Se-Al v.2.0a11 (Rambaut 2007), which allowed the alignment to be exported and saved in FASTA format. Maximum likelihood analysis was performed on the Sinningia nrITS alignment with MEGA 5.03 for Mac OS X (Tamura et al. 2011), using the Tamura–Nei substitution model with gamma distribution (TN93 + G) to estimate rate variation across nucleotide sites. Reliability of the phylogenetic tree was estimated by BS with 1000 replications.

Results

AFLP

Sixteen AFLP primer pairs were initially screened against a set of six S. speciosa genotypes, and four that gave a relatively uniform peak distribution were chosen and were informative in the group of test samples (data not shown). Amplified fragment length polymorphism profiles were generated for the 26 Sinningia accessions using the E + 3/M + 3 primer combinations E32M48, E32M50, E33M49 and E33M50. Amplified fragment length polymorphism peaks were scored as 1 = present, 0 = absent. Of the 602 scored fragment bins, 591 (98.2 %) were polymorphic in this sample set. The 11 monomorphic bins were removed, as were four that were outside the range of the size standards. Within the 24 accessions of S. speciosa (S. guttata and S. macrophylla removed) there were 563 bins, of which 558 (99 %) were polymorphic (Table 2). The final data matrix consisted of 15 264 binary scores (26 accessions × 587 bins). A Dice/UPGMA phenogram derived from the AFLP data is shown in Fig. 3. The cophenetic correlation coefficient (normalized Mantel statistic Z) was $r = 0.94651$, indicating that the tree was significantly
correlated with the similarity matrix from which it was computed. Bootstrap values for all nodes present in the consensus tree ranged from 51 to 100 %, with a mean of 88.8 %. The same analysis performed using Jaccard’s coefficient (Jaccard 1908) produced a very similar but more complicated tree with a slightly higher correlation coefficient ($r = 0.95526$) and identical BS percentages for the conserved nodes (not shown).

To estimate the level of inherent error in the AFLP procedure, four independent AFLP amplifications were performed on DNA from $S$. speciosa ‘Guatapara’ that had been digested with EcoRI and MseI. Adaptor

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**Table 2** Number, sizes and relative polymorphism of AFLP markers amplified from *Sinningia* genomic DNA with four primer combinations. In determining the number and per cent of mono- or polymorphic AFLP markers, ‘all *Sinningia*’ includes the wild and cultivated accessions of *S.* speciosa as well as one accession each of *S.* guttata and *S.* macrophylla.

| Primer pair     | E32M48 | E32M50 | E33M49 | E33M50 | Totals |
|-----------------|--------|--------|--------|--------|--------|
| No. of bins     | 115    | 155    | 130    | 202    | 602    |
| Mean no. of peaks/entry | 22.7   | 41.2   | 34.6   | 64.2   |
| Size range (bases) | 60–638 | 59–552 | 57–671 | 60–690 |
| Number/% monomorphic (all *Sinningia*) | 3/2.6  | 2/1.3  | 1/0.8  | 5/2.5  |
| Number/% polymorphic (all *Sinningia*) | 112/97.4 | 153/98.7 | 129/99.2 | 197/97.5 |
| Number/% monomorphic (*S. speciosa*) | 3/2.8  | 4/2.6  | 2/1.6  | 7/3.6  |
| Number/% polymorphic (*S. speciosa*) | 103/97.2 | 148/97.4 | 125/98.4 | 188/96.4 | 564/97.2 |

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**Fig. 3** Dice/UPGMA tree showing phenetic relationships within a group of 24 *S. speciosa* accessions (17 wild and seven cultivars) based on AFLP data. Two related species, *S. guttata* and *S. macrophylla*, were also included in the analysis. Major clusters A and B are shown in pink and blue, respectively. Secondary clusters A1 and A2 are labelled at the appropriate nodes. Tertiary clusters A1-1, A1-2, A2-1, A2-2 and A2-3 are indicated by brackets. Bootstrap percentages $>50 \%$ are shown adjacent to the cluster nodes.
lifications, PSA reactions with primers E01 and M01, and SA reactions with primer pair E32M50 were carried out as described earlier (Zhang et al. 2012). Six replicates of each SA were then separated on the CEQ8000 instrument, and the data analysed as for the experimental samples. The E32M50 primer pair gave a total of 76 fragment bins between 67 and 570 nucleotides. This included three bins with only a single entry (unique peaks), which were removed from the analysis. Pair-wise similarities (simple matching) for the six within-group replicates were 0.945–0.986 (group 1), 0.959–0.973 (group 2), 0.973–1.00 (group 3) and 0.973–1.00 (group 4). This translates into error rates of 0–5.5 %, which is within the range cited by Meudt and Clarke (2007) for other plant AFLP studies. There were 17 peaks that were either present or absent for all entries within one or more replicate sets. Examination of the chromatograms revealed that this was due to minor between-run variances in the instrument scoring, which resulted in the peaks being placed in a new bin $\pm 1$ nucleotide of the expected size, and was readily corrected.

Dice/UPGMA analysis of the AFLP data provided evidence of genetic diversity within S. speciosa. Two major clusters were evident in the phenetic tree, with 20 of the 26 accessions present in cluster A (although there is a major division within this large cluster) (Fig. 3). The placement of ‘Serra da Vista’ could imply the existence of a third cluster, although its position between clusters A and B makes its affiliations inconclusive. Cluster A1 consisted of three plants collected from Rio de Janeiro state (AC1503, ‘Buzios’ & ‘Cabo Frio’), one plant from the southern part of Minas Gerais (‘Carangola’) and three plants from Espirito Santo state (‘Espírito Santo’, ‘Santa Teresa’ and ‘Domingos Martins’). Cluster A2 contained two plants from Rio de Janeiro (‘Avenida Niemeier’ and ‘São Conrado’), the unidentified wild accession ‘CM’, and all of the domesticated varieties (‘Kaiser Wilhelm’, ‘Dona Lourdes’, ‘Guatapara’, ‘Purple’, ‘Santa Teresa’, and ‘Regina’ in subcluster A1-2. Principal coordinates analysis also separated the three cultivars with wild-type flowers (‘Dona Lourdes’, ‘Guatapara’ and ‘Purple’) from the peloric cultivars + wild collections in cluster A2. This discrete subcluster was preserved from the Dice/UPGMA tree and is designated A2-2 (Fig. 3).

Genetic distance estimates derived from the AFLP data were plotted against calculated geographic distances for all pairwise combinations of wild S. speciosa accessions. The unverified collections ‘Jurapé’, ‘CM’ and ‘Massaguassu’ were excluded from this analysis. Linear regression of the data shows a positive relationship between genetic and geographic distances for S. speciosa in south-eastern Brazil (Fig. 5). The Mantel test, as implemented in IBDWS version 3.23 (Bohonak 2002; Jensen et al. 2005), indicated that the matrices of genetic and physical distances were significantly correlated ($Z = 7525.992$, $r = 0.3530$, $P \leq 0.0053$). This relationship held for all combinations of arithmetic and logarithmic values derived from the AFLP data, but not for GD estimates calculated from phylogenetic comparisons of the nrITS sequences (not shown).

**Sinningia nrITS sequences**

The full intergenic ITS1-5.8S-ITS2 region was amplified from genomic DNA isolated from the 26 Sinningia accessions using primers complementary to conserved sequences at the 3′-end of the 18S and the 5′-end of the 26S rRNA genes (for a map of this region in angiosperms, see Hershkovitz et al. 1999). Unambiguous nucleotide sequences were obtained directly from all amplified fragments with the exception of that from S. speciosa ‘Kaiser Wilhelm’, which was cloned prior to sequencing. There was no indication of paralogous loci or pseudogene amplification from any accession used in this study (see Baldwin et al. 1995; Buckler et al.}
The criteria used by Besnard et al. (2009) suggested that the sequenced nrITS regions are part of functional rDNA repeats (Table 3). In addition, the conserved 14 bp sequence (5'-GAATTGCAGAATCC-3') that is diagnostic of plant 5.8S RNA genes (Jobes and Thien 1997) was invariant in all Sinningia ITS region sequences. Based on comparisons with published ITS sequences from carrot and broad bean (Yokota et al. 1989), the S. speciosa ITS1–5.8S–ITS2 region was determined to be 622 bp long, and the lengths of the two spacers were 236 bp (ITS1) and 222 bp (ITS2). The lengths of the nrITS regions reported here for Sinningia are comparable to those from many other species of angiosperms.
The highest level of sequence divergence for the 27 nrITS sequences was 7.9% between S. speciosa ‘Carangola’ and S. guttata. Within the group of 17 wild S. speciosa collections, divergence ranged from 0 to 4% (Table 3), which is comparable to levels of intraspecific nrITS divergence previously reported in angiosperms, e.g. 3.7 and 1.2–1.8%, respectively, between subspecies of Calycadenia truncata (Baldwin 1993) and Caulanthus amplexicaulis (Pepper and Norwood 2001), 0–5.2% between members of the Streptanthus glandulosus complex (Mayer and Soltis 1999), 0.4–4.9% within several populations of the Arctic endemic Saxifraga cernua (Brochmann et al. 1998) and an average of 2.4% between European and North African populations of Saxifraga globulifera separated by the Straits of Gibraltar (Vargas et al. 1999). Higher levels of intraspecific nrITS divergence, up to 7.68% for ITS1 + ITS2, have been reported for species of Aeschynanthus (Gesneriaceae; Denduangboripant et al. 2001), and could very

Table 3 Features of the nrITS region from Sinningia. Internal transcribed spacer-specific fragments were amplified from genomic DNA isolated from 24 accessions of S. speciosa and one each of S. guttata and S. macrophylla using the primers ITS6 and ITS8m. The lengths of ITS1 and ITS2 were determined by comparison with sequences from carrot and broad bean (Yokota et al. 1989). Sequence analysis was performed with DnaSP v5 (Librado and Rozas 2009). RNA secondary structure minimum free energies (∆G) were calculated individually for ITS1 and ITS2 from all nrITS sequences using the Mfold web server (http://mfold.rna.albany.edu/) (Zuker 2003).

| nrITS sequence | Overall length | 622–623 bp |
|----------------|----------------|-------------|
| Aligned length | 624 bp (2 gaps) |
| 5.8S rDNA length | 164 bp |
| Mean G + C content | 53% |
| No. of variable sites | 85 |
| Parsimony informative sites | 33 |
| Maximum sequence divergence (no./%) (all Sinningia) | 49/7.9 (S. guttata—‘Carangola’) |
| Maximum sequence divergence (no./%) (wild S. speciosa only) | 25/4.0 (‘Carangola’—‘Espírito Santo’) |
| Maximum sequence divergence (no./%) (all S. speciosa) | 27/4.3 (‘KW-C’—‘Espírito Santo’) |
| Minimum free energy (∆G) kcal mol⁻¹—ITS1 | −78.30 to −90.60 (mean = −86.98) |
| Minimum free energy (∆G) kcal mol⁻¹—ITS2 | −75.90 to −90.50 (mean = −81.40) |

(Baldwin et al. 1995; Vander Stappen et al. 1998; Vargas et al. 1999; Schnabel et al. 2003), including sequences from both New and Old World Gesneriaceae (Möller and Cronk 1997; Zimmer et al. 2002; Hughes et al. 2005; Roalson et al. 2005). The 5.8S rRNA gene was 164 bp in length in all accessions of Sinningia examined, which is within the range of values (160–165 bp) published for other angiosperms (Baldwin 1993; Baldwin et al. 1995; Vander Stappen et al. 1998; Desprès et al. 2003; Pelser et al. 2003; Schnabel et al. 2003). The conserved 21 bp sequence 5′-GGGC GCCAACAGC CGCA-AGGAA-3′, possibly important in RNA processing (Liu and Schardl 1994), was present in ITS1 at positions 140–160. The aligned data matrix for all full-length nrITS sequences was 624 bp, which included 1 bp gaps in ITS1 and ITS2 at positions 93 and 447, respectively. The individual alignments were 237 bp (ITS1) and 223 bp (ITS2) in length. There were 33 (5.3%) parsimony-informative sites in the alignment, 16 in ITS1, 14 in ITS2 and three in the 5.8S RNA gene. Of the 52 singleton sites, 18 were in ITS1, one was in the 5.8S gene and 33 were in ITS2. Eighty-six per cent (537/624) of the sites were conserved. There was no missing data in the alignment.
well result from reduced rates of concerted evolution at the ribosomal repeat loci (Denduangboripant and Cronk 2000).

Maximum likelihood analysis with BS was performed on the Sinningia nrITS alignment. The resulting unrooted tree with nodal BS values is shown in Fig. 6. Maximum likelihood separated the 27 ITS sequences into two major clades, with S. guttata as outgroup. Clade #1, which contained all of the cultivated forms and six wild collections, had strong statistical support (98 % BS), as did the three smaller subclades 2-1, 2-2 and 2-3 (BS values ≥ 80 %). The best support was for the small clade 2-3 (99 % BS), which was nearly identical to cluster A1-1 from the Dice/UPGMA phenogram.

Discussion

Cluster analysis of AFLP data

A Dice/UPGMA cluster analysis was performed on the Sinningia AFLP data matrix to uncover possible genetic structure within a group of 24 accessions of S. speciosa. Included as outgroups were one accession each of S. guttata and S. macrophylla, which are phylogenetically close to S. speciosa (Perret et al. 2003) and are the only...
species with which it will form fertile hybrids. Phenetic cluster analysis groups individuals or species based on presently existing shared characters, which in this case are genomic DNA fragments. Phenetics is distinguished from cladistics, which seeks to reconstruct evolutionary relationships from DNA sequence alignments.

Amplified fragment length polymorphism markers are anonymous DNA fragments that are amplified from loci distributed randomly throughout the genome. The individual fragments—here detected as fluorescent peaks—are coded as binary markers (1 = present, 0 = absent) and scored as independent unit characters of equal weight. The presence of the null allele (0) makes it impossible to distinguish between the allelic states 1/1 and 1/0 in most cases, and thus AFLPs mimic dominant morphological genetic markers. Amplified fragment length polymorphism fragments are amplified under very selective conditions—each fragment is flanked by two restriction enzyme recognition sites (EcoRI and MseI in this case), and the amplification specificity is determined by the six selective nucleotides (3 + 3) internal to the restriction sites that extend beyond the 3′-end of each primer. Therefore, while a single set of criteria results in successful amplification of any given fragment, amplification failure (fragment absence) can be attributed to at least 18 distinct events: a change (mutation) in one or more of the 16 critical nucleotides (6 for EcoRI + 4 for MseI + 6 selective), or an insertion or deletion event (indel) that encompasses either of the terminal restriction sites or the sequence between them. Subsequent analyses could potentially be confounded because it is impossible to distinguish between any of the multiple events that lead to fragment absence. Dice’s similarity coefficient, which is equivalent to 1 minus Nei and Li’s GD (Nei and Li 1979), and Jaccard’s coefficient are well suited for the analysis of AFLP data because both only consider shared characters (scores of 1) within each fragment bin, and give no weight to the shared absence of a band (including shared band absence would introduce homoplasy into the data set for the reasons given above). These methods are advantageous because band absences (scores of 0) are excluded from the analyses, and there are also no assumptions of the Hardy–Weinberg equilibrium. Phenetic analysis is therefore appropriate for use with AFLP data (Koopman et al. 2001; Spooner et al. 2005).

Scoring of non-homologous fragments (co-migrating fragments that have different sequences) is one potential source of homoplasy that can introduce error into distance estimates calculated from AFLP data (Després et al. 2003; Meudt and Clarke 2007). However, the few studies that have addressed this issue through DNA sequencing found a high degree of identity in the sequenced AFLP fragments (Simmons et al. 2007). At lower taxonomic levels, AFLP fragment-size homoplasy will be minimal, and can be expected to have little effect in intraspecific studies (Tremetsberger et al. 2006). Indeed, recent computer simulations based on sequenced genomes indicated that homologies of co-migrating AFLP fragments are greater between closely related taxa, and that fragment-size homoplasy is much lower in organisms with small (<400 Mbp) versus large (>2 Gbp) genomes (Althoff et al. 2007).

The intent of this study was to examine intraspecific relationships within a group of wild collections and cultivars of S. speciosa. In the Dice/UPGMA dendrogram (Fig. 3), the S. speciosa accessions were divided into two major clusters (A and B). Within cluster A, there was a major division that essentially separated all of the cultivars from the majority of the wild collections (secondary clusters A1 and A2). These two subclusters were further subdivided into two (A1 and three (A2) well-supported tertiary clusters. Cluster A1-1 contained three plants collected in the Cabo Frio region of south-eastern Rio de Janeiro state (AC1503, ‘Buzios’ and ‘Cabo Frio’) and had very high statistical support. The very close identity with ‘Cabo Frio’ implies that the commercial accession ‘Chiltern Seeds’ also originated in that area. The four members of cluster A1-1, including ‘Espirito Santo’, are united by their common morphology in that they are relatively small plants with distinctive bright green foliage and small lavender flowers. Cluster A1-2, which is a sister to A1-1, contains three accessions (‘Domingos Martins’, ‘Regina’ and ‘Santa Teresa’) that have dark-coloured leaves with silver veins and purple flowers. ‘Regina’ is of unknown origin (Sprague 1904), while both ‘Domingos Martins’ and ‘Santa Teresa’ originated in Espirito Santo state. ‘Carangola’, a unique form with bi-coloured flowers from Minas Gerais, also resides in cluster A1-2, although the BS support for this relationship is only 61 %. Cluster A2 contained both wild and cultivated accessions of S. speciosa. The four wild collections in A2 all grouped together in subcluster A2-1; ‘Avenida Niemeyer’ and ‘São Conrado’ were both collected close to the beach in the southern part of Rio de Janeiro, and are larger plants with large, dark purple flowers. ‘Jurapé’ is an unconfirmed collection from Santa Catarina state that showed affinity with ‘CM’, although the BS support (51 %) was relatively weak. Considering that the distance between Rio de Janeiro and the closest point in north-eastern Santa Catarina state (Joinville) is >900 km, the purported geographical origin of this collection could be in doubt. ‘Kaiser Wilhelm’, a peloric ‘gloxinia’ cultivar from the late 19th century, was the single cultivated accession...
in cluster A2-1. Clusters A2-2 and A2-3 contained only cultivated accessions, and it is interesting that the three cultivars with nodding (wild-type) flowers, ‘Dona Lourdes’, ‘Purple’ and ‘Guatapara’, grouped together in cluster A2-2 (BS = 99%). The three remaining cultivars in cluster A (JFS4512, ‘Ken’s White’ and ‘Red with Spots’) all have peloric flowers and formed a well-supported subcluster (A2-3; BS = 85%). Cluster B (BS = 99%) contained S. macrophylla and three wild collections of S. speciosa—‘Antônio Dias’, Cardoso Moreira-pink’ and ‘Massaguassu’. ‘Antônio Dias’ and ‘Cardoso Moreira-pink’ have elongated stems and are morphologically distinct from the other wild forms of S. speciosa included in this study.

Principal coordinates analysis was also used to visualize the relationships within the group of 26 Sinningia accessions. Principal coordinates analysis is an ordination method similar to the principal components analysis, and both are frequently applied to the exploration of AFLP data (Koopman et al. 2001; Kim et al. 2004; Chao et al. 2005; Fernandez et al. 2006; Azizi et al. 2009). Cluster assignments from the Sinningia Dice/UPGMA tree (Fig. 3) were preserved in the PCoA (Fig. 4), and they supported the results of the AFLP phenetic analysis. Two minor differences were noted between the two analyses: (i) in the PCoA, AC1503 is somewhat removed from the other members of cluster A1-1 (‘Buzios’, ‘Cabo Frio’, ‘Chiltern Seeds’ and ‘Espírito Santo’), and (ii) ‘Serra da Vista’ is included in PCoA cluster A1-2, whereas it was placed between the major clusters A and B in the Dice/UPGMA tree. Principal coordinates analysis also shows that the plants in cluster B do not appear to be closely affiliated with the other S. speciosa accessions.

Phylogenetic analysis of the nrITS region in S. speciosa

A phylogenetic analysis of the nrITS region was also performed for S. speciosa. The nrITS sequence is a popular tool in plant systematics, and has been used for investigating species relationships in both Old World and New World Gesneriaceae (Möller and Cronk 1997; Zimmer et al. 2002; Clark and Zimmer 2003; Roalson et al. 2005; Clark et al. 2009; Wang et al. 2011). The nrITS region is most informative at higher taxonomic levels (species and above), although intraspecific ITS sequence variation has been documented in several angiosperm dicot families such as the Apiaceae (Vargas et al. 1998), Asteraceae (Baldwin 1993), Brassicaceae (Mayer and Soltis 1999; Pepper and Norwood 2001), Fabaceae (Vander Stappen et al. 1998), Saxifragaceae (Broomham et al. 1998; Vargas et al. 1999), Lamiaceae (Prather et al. 2002) and Gesneriaceae (Hughes et al. 2005). In the present study, the nrITS region (ITS1–5.8S–ITS2) was amplified from the same 26 Sinningia accessions included in the AFLP analysis. Unambiguous nucleotide sequences were obtained for 25 accessions directly from the purified PCR products. Repeated sequencing failures necessitated cloning of the amplified fragments from ‘Kaiser Wilhelm’, however. Sequencing of several recombinant plasmids revealed that the ITS region is polymorphic in this particular cultivar, and sequences from two representative clones were included in the alignment. Intra-individual nrITS sequence polymorphism can result from incomplete concerted evolution, the process by which repetitive sequences in a gene family become homogenized through gene conversion (Liao 2008). Incomplete concerted evolution of the rRNA gene repeats is suggested in the ancient gymnosperm Cycas, due to the presence of highly polymorphic functional nrITS repeats as well as many divergent ITS paralogues isolated from individual plants (Xiao et al. 2010). Incomplete concerted evolution is also apparent in taxa of hybrid origin in Rosaceae (Campbell et al. 1997; Ritz et al. 2005), and in many species of Aeschynanthus, a genus of south-east Asian Gesneriaceae (Denduangboripant and Cronk 2000).

The ML tree (Fig. 6) shows that the nrITS DNA sequence phylogenetically informative in S. speciosa, although several nodes had BS support of <50%. The most noteworthy feature of the phylogram is that many of the clusters present in the AFLP analyses are retained in the ML tree. Significantly, all of the ‘gloxinia’ cultivars—regardless of flower form—grouped together in one large, well-supported clade (clade #1) along with the two known collections from Rio de Janeiro state (‘Avenida Niemeyer’ and ‘São Conrado’). The two ITS sequences from ‘Kaiser Wilhelm’ reside in this clade, even though they differ at five sites each in ITS1 and ITS2 (nine transitions and one transversion). There is also a 1 bp insertion (T) in KW-C ITS1, which probably accounted for the difficulty sequencing the mixed ITS amplicons directly. Pairings between ‘Avenida Niemeyer’ and ‘São Conrado’ and between ‘Ken’s White’ and JFS4512 are retained from the AFLP tree, and clade #1 is nearly equivalent to Dice/UPGMA cluster A2. Major differences are the inclusion of ‘Carangola’ and ‘Massaguassu’ in this clade. Also retained in the ML tree is the ‘Antônio Dias’–‘Cardoso Moreira-pink’ pairing in clade 2-2, and the close relationship between the three collections with dark leaves and silver veins (‘Regina’, ‘Domingos Martins’ and ‘Santa Teresa’) in clade 2-1.

Conclusions and forward look

Independent phenetic and phylogenetic analyses of two different data sets (AFLPs and nrITS DNA sequences,
respectively) show that the available wild collections of S. speciosa comprise a genetically diverse group; estimates of GD calculated from the AFLP data are positively correlated with geographic distance, indicating that the observed diversity could result from reproductive isolation. Unfortunately, there has been no detailed population survey conducted for S. speciosa, and its present geographical range is poorly delimited at best. In addition, some of the wild collections included here (e.g. ‘Jurapé’ and ‘Massaguassu’) came with anecdotal collection information. For these reasons, this study must be considered preliminary. Nevertheless, several conclusions can be drawn from the results presented here. (i) All of the ‘gloxinia’ cultivars reside in cluster A2 and clade #1, evidence that the primary gene pool for the cultivars probably came from plants originating in Rio de Janeiro. This makes sense, because historical records state that several of the early ‘gloxinias’ exported to Europe were collected within or near this city (Hooker 1842; Paxton 1846; Brackenridge 1886). However, the separation of three of the erect-flowered cultivars in cluster A2-3 (Fig. 3) could be an indication that plants from at least one other geographic region contributed genetically to the cultivars grown today. (ii) Plants collected in the Eastern Cape region of Rio de Janeiro state and very southern Espírito Santo state form a cohesive group. These plants are distinct from those found ~160 km to the west of the city of Rio de Janeiro and also from plants collected in eastern Minas Gerais and north-eastern Espírito Santo states. (iii) The unknown wild collection ‘Regina’ groups with morphologically similar plants having dark leaves with silver veins in phenetic cluster A1-2 and phylogenetic clade #2-1, and was therefore most probably collected in Espírito Santo state. (iv) The three cultivars with wild-type (nodding) flowers (‘Dona Lourdes’, ‘Guatapara’ and ‘Purple’) in AFLP subcluster A2-2 are distinct from the other cultivars with peloric (erect) flowers. ‘Dona Lourdes’ and ‘Guatapara’ have much larger nuclear genomes than any other accessions of S. speciosa (Zaitlin and Pierce 2010) and appear to be tetraploids, which could be one factor to account for the observed separation from the other cultivars. (v) The wild collection ‘Jurapé’ is almost certainly not from Santa Catarina state, since both analyses group it with plants from Rio de Janeiro and the cultivars. (vi) The taxonomic position of S. macrophylla has a very restricted range in the dry forests of southern Bahia, well isolated from known populations of S. speciosa (Thomas 2007; Araujo and Chautems 2010). In the present study, the phenetic analysis places S. macrophylla in AFLP cluster B with several divergent forms of S. speciosa (Figs 3 and 4), and it is nested within S. speciosa in clade #2 in the ML tree (Fig. 6). Further work using molecular markers (see below) and/or other DNA sequences will be required to clarify the relationship between these two taxa. Sinningia speciosa is not listed in the IUCN Red List of Threatened Species (http://www.iucnredlist.org), although many other unlisted species of Sinningia meet the criteria for inclusion in the endangered or critically endangered IUCN categories (Chautems et al. 2010). Sinningia speciosa is distributed over a relatively large area, but has almost certainly experienced considerable habitat disruption given that the Mata Atlântica presently exists in ~245 000 forest fragments, many of which are small, <100 ha in size (Ribeiro et al. 2009). Recent observations of S. speciosa in the field in Brazil (D.Z., December 2011) revealed that (i) populations are often isolated and may consist of very few individuals; (ii) populations do not overlap (they are not sympatric); (iii) many populations grow outside of protected areas, sometimes adjacent to agricultural enterprises; and (iv) the various diverse morphological forms are locally endemic, and some are quite rare. For these reasons, there is an urgent need to discover, describe and systematically sample wild populations of S. speciosa for genetic diversity. This laboratory is presently developing a suite of co-dominant genetic markers based on simple sequence repeats (Morgante et al. 2002) and intron sequences from annotated genes. These markers are highly informative, and will be invaluable for genotyping wild and cultivated germplasm, and for evolutionary studies in S. speciosa and its relatives. More importantly, we will be able to quantify the levels of allelic diversity and the extent of gene flow within and between populations of S. speciosa. This in turn will allow us to define evolutionarily significant units (Moritz 2002), and make recommendations for conservation of this interesting and unique species.

**Additional information**

The following additional information is available in the online version of this article –

File 1: Large population of S. speciosa growing on a granite wall along the coastal road Avenida Niemeyer in the southern part of Rio de Janeiro, Brazil. Photograph taken on 27 November 2011 by D. Zaitlin.
File 2: A population of *S. speciosa* growing on a slope adjacent to the sea near the town of Armação dos Búzios, RJ, Brazil. Photograph taken on 28 November 2011 by D. Zaitlin.

File 3: A dark-leaved form of *S. speciosa* growing on a moist bank above a dirt road near Córrego da Xica, RJ, Brazil. Photograph taken on 28 November 2011 by D. Zaitlin.

File 4: A population of a dark-leaved form of *S. speciosa* with bi-coloured flowers growing adjacent to a stream near the Hotel Fazenda Pedra Lisa, RJ, Brazil. Photograph taken on 29 November 2011 by D. Zaitlin.

File 5: A caulescent form of *S. speciosa* growing at the top of a hill with cacti and spikemosses near the town of Cardoso Moreira, RJ, Brazil. Photograph taken on 29 November 2011 by D. Zaitlin.

Accession numbers
DNA sequences of the *Sinningia* nrITS region (ITS1 + 5.8S RNA gene + ITS2) used in this study have been deposited with GenBank. Accession numbers are: JQ928140 (*S. macrophylla*), JQ928141 (*S. guttata*) and JQ928142 to JQ928165 (wild and cultivated *S. speciosa*).

Sources of funding
The author acknowledges Kentucky Tobacco Research and Development Center for providing laboratory space and scientific infrastructure, and Dr H.M. Davies and the Kentucky Tobacco Research Board for supporting this research.

Acknowledgements
I thank Dr Kermit Ritland and an anonymous reviewer for their detailed reading of the manuscript and their kind assistance with editorial suggestions. I am also greatly indebted to my colleague Alain Chautems of the Conservatoire et Jardin Botaniques de la Ville de Genève, with whom I have had many interesting and productive discussions about *Sinningia speciosa* and other Brazilian species of Gesneriaceae.

Conflict of interest statement
None declared.

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