Phylogenetic Analysis and Karyotype Evolution in the Genus Clivia (Amaryllidaceae)

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Received: 12 December 2000 Returned for revision: 31 January 2001 Accepted: 12 March 2001

Phylogenetic relationships of five taxa of Clivia, one probable new species plus four recognized species, and three outgroup species were studied using sequences of the nuclear ribosomal 5S non-transcribed spacer and the internal transcribed spacer (ITS) of 45S rDNA. Analysis of the data sets separately generated some well-supported groupings and congruent phylogenies. Clivia miniata and C. gardenii are closely related. ‘Robust Gardenii’, the putative new species, is a sister clade of this group. Clivia nobilis is faintly related to these three taxa and C. caulescens occupies an intermediate position between the two groups. Chromosome locations and distribution patterns of the 5S nuclear ribosomal gene in the species of Clivia were investigated using fluorescence in situ hybridization (FISH). In all species, only one pair of 5S rDNA signals was observed. These were located on the short arm of chromosome 8, at the position of the interstitial C-bands. The phylogenies obtained from the DNA sequences together with the chromosome data accumulated here and previously published information on the location of the 45S rDNA sites have been used to postulate evolutionary trends in Clivia chromosomes.

Key words: Clivia, chromosome evolution, 45S and 5S rDNA, ITS, FISH, molecular phylogeny.

INTRODUCTION

The genus Clivia Lindl. is a member of the Amaryllidaceae and includes four named species primarily distributed in eastern South Africa. Three of the species were discovered and described in the 19th century. Since then they have been cultivated as ornamentals, with C. miniata Regel being the most widely grown (Duncan, 1999). With the exception of the slow-growing C. nobilis Lindl., the vegetative morphology of the species is somewhat similar. In addition to the four named species, a proposed new species, here called ‘Robust Gardenii’, is also included in this analysis. It has been identified as a result of our previous studies using karyotype analysis and RAPD markers in addition to field observations on the morphology of both wild and cultivated plants (Ran et al., 1999; Ran, 2000). A formal description of the new species will be published elsewhere.

Recent studies on the karyotypes of the genus (Ran et al., 1999) have shown that all species have the same chromosome number (2n = 2x = 22) and basic chromosome morphology. However, when differential staining techniques, such as Giemsa C-banding, CMA- and Ag-NORs banding were used, variation between the species was observed. Each species was found to have at least one unique band. The major distinguishing features were firstly, the presence in C. miniata, C. gardenii Hooker and ‘Robust Gardenii’ of prominent centromeric bands on all chromosomes and their complete absence in C. nobilis and C. caulescens Dyer and, secondly, the number of 45S rDNA sites (Ran et al., 1999). Clivia gardenii has a single pair of 45S rDNA sites, C. miniata, C. caulescens and ‘Robust Gardenii’ have two pairs and C. nobilis three pairs. It is usually difficult to discern the direction of karyotype change in plants but with the development of techniques for rapid DNA sequencing it is now possible to produce phylogenetic trees that may be used for evolutionary analysis.

The internal transcribed spacers (ITS1 and ITS2) of nuclear ribosomal 45S DNA (rDNA) are non-coding regions of relatively rapidly evolving DNA sequences that flank the very slowly evolving 5.8S rDNA genes. These sequences have been used extensively for phylogenetic inference among relatively closely related species (Baldwin et al., 1995). The non-transcribed spacer between the 5S rRNA genes is also rapidly evolving and has also been used successfully to produce phylogenies in a number of species (Udovicic et al., 1995; Crisp, 1999; Persson, 2000). However, in some plant species the variation between the repeated units of an individual array makes the 5S rRNA gene spacer difficult to use in phylogenetic studies (Baum and Johnson, 1999). This variation may be due to lack of interlocus concerted evolution for 5S rDNA arrays in these plants (Cronn et al., 1996).

In this study we utilized these rDNA spacer regions to construct phylogenies of Clivia then used the phylogenies to analyse patterns of chromosome evolution in the genus.

MATERIALS AND METHODS

The plant material used in this study is listed in Table 1. Two plants of ‘Robust gardenii’, which we recognize as distinct from the four named species, were included in the analysis, together with two different plants of each of the

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0305-7364/01/060823 + 08 $35.00/00
named species. Using the classifications of Traub (1963) and Meerow et al. (1999), three species from closely related genera in the Amaryllidaceae were selected as outgroup species. Haemanthus coccineus L. and Cryptostephanus vansonii Verdoorn were used for the two ITS regions and Lycoris aurea L’Hérit. was used for the 5S rRNA gene spacer due to the occurrence of multiple bands following PCR amplification of the 5S genes in the former two species.

Chromosome preparation, probe DNA labelling, in situ hybridization and C-banding

Root tips were pretreated with a saturated solution of paradichlorobenzene (PDB) for 18–24 h at 4 °C, fixed in freshly prepared 3:1 (v/v) absolute ethanol:glacial acetic acid for at least 24 h at 4 °C and stored in 70 % ethanol at 4 °C. Chromosome spreads were made using the modified enzyme digestion method described by Ran et al. (1999).

The plasmid p55Pr10 which contains 5S rDNA of *Pinus radiata* D. Don (Jacobs et al., 2000) was labelled with biotin-16-dUTP by nick translation following the manufacturer’s instruction (Boehringer Mannheim, Mannheim, Germany) and used to detect sites of the 5S rRNA genes. Fluorescence in situ hybridization (FISH) and C-banding were carried out using the procedure described by Ran et al. (1999). Photographs of FISH preparations were taken with Fujichrome PROVIA 400 colour film. The images were then digitized with a Nikon LS 1000 film scanner and the whole images were merged using Adobe Photoshop 3.0.5.

DNA isolation and polymerase chain reaction (PCR)

DNA for PCR was extracted from fresh root tips using the Nucleon Phytopure kit (Amersham Pharmacia Biotech., Little Chalfont, Bucks, UK). The ITS1 and ITS2 regions

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### Table 1. Species, source and GenBank accession numbers of the plants used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Voucher</th>
<th>Source</th>
<th>ITS region</th>
<th>5S rDNA repeat</th>
</tr>
</thead>
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<tr>
<td>C. caulescens</td>
<td>K Hammett 880 (AKU)</td>
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<td>687 GBAN AF324753</td>
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<td>GBAN AF324754</td>
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<td>Cultivated population, via Japan</td>
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<td>687 GBAN AF324756</td>
</tr>
<tr>
<td></td>
<td>K Hammett 827 (AKU)</td>
<td>South Africa, via UK (wild)</td>
<td>648 GBAN AF324024</td>
<td>687 GBAN AF324755</td>
</tr>
<tr>
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<td>Kirstenbosch, South Africa (wild)</td>
<td>648 GBAN AF324025</td>
<td>687 GBAN AF324757</td>
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<td>C. miniata</td>
<td>K Hammett 886 (AKU)</td>
<td>Howick, South Africa, (wild)</td>
<td>648 GBAN AF324026</td>
<td>687 GBAN AF324758</td>
</tr>
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<tr>
<td></td>
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<td>K Hammett 8661 (AKU)</td>
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<td>GBAN AF324764</td>
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<td>Garden origin</td>
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<td>GBAN AF324765</td>
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<tr>
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<td>Thai/Burma border (wild)</td>
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![Diagram showing the organization of 45S and 5S rDNA repeats and primer positions for PCR.](image-url)
were amplified individually for high yield using standard double-stranded PCR with primers ITS2, ITS3, ITS4 and ITS5 (Fig. 1) (White et al., 1990). The PCR procedure was carried out as described by Gatt et al. (1999). Complete 5S repeat products were obtained by PCR using two sets of primer. One set was 5SR (5'-CACCACCGATCATCGAATC-3') and 5SF (5'-TTAGTCTGGATGCTGCA-3') (Udovicic et al., 1995). The other set designed specifically for Clivia was 5SPI (5'-GAGTTCTGATGGGATCCGGTG-3') and 5PP2 (5'-TGCTTGGGAGAGCATCTTGT-3'). The positions of these primers on the 5S repeat are shown in Fig. 1. The PCR reaction was carried out in 25 µl tubes containing 1 × PCR reaction buffer, 0.2 mM dNTPs, 2 mM MgCl₂, 0.25 µM each of primer, 1.25 U Taq polymerase (Boehringer Mannheim) and 30 ng template DNA. Reaction conditions followed Brown and Carlson (1997) and the products were separated on an agarose gel. Single bands were obtained from C. miniatu, C. gardenii, ‘Robust Gardenii’ and C. caulescens, double bands from C. nobilis and the outgroup Lycoris aurea.

Individual bands were excised from the gels and DNA extracted using the Gel Extraction Kit (Qiagen, Valencia, CA, USA).

Cloning of PCR product of 5S spacer

The PGEM-T vector (Promega, Madison, Wisconsin, USA) was used for cloning PCR products of 5S rDNA. Ligation of the vector and 5S rDNA was made using ligase in 1 × ligation buffer (Promega), followed by incubation at 4 °C for 24 h. Transformation was carried out following a standard protocol (Promega Technical Manual) using competent Escherichia coli. Bacteria were cultured in SOC medium at 37 °C for 1 h and then on LB agar plates containing 100 µg ml⁻¹ ampicillin, 100 mM IPTG and 2 % X-gal at 37 °C for 24 h, which allowed selection of recombinant transformants. Ten randomly selected clones from each species were cultured in 3 ml LB with 100 µg ml⁻¹ ampicillin. Minipreparation of plasmid DNA was made with the Qiagen Minipreparation Kit, followed by

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Fig. 2. Maximum likelihood trees generated from the entire ITS region (A) and 5S rDNA spacer (B) using GTR and K2P distance methods, respectively. The values above branches are percentages of how often the corresponding cluster was found among 1000 intermediate trees using the quartet puzzling test. Neighbour-joining trees produced from the entire ITS region (C) and 5S rDNA spacer (D) using GTR and K2P distance methods, respectively. The values above branches are bootstrap values based on 1000 replicates.
restriction analysis, allowing identification of clones for sequencing.

DNA sequencing

Automated sequencing of the purified double-stranded PCR products and plasmid clones was carried out in both directions. Thermal cycling reactions were performed on a Perkin Elmer Geneamp PCR System 2400, using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS following the manufacturer’s protocol. Sequencing was then done on a 377 ABI Stretch Sequencer. BLAST searches confirmed the plant origin of our products.

Sequence alignment

Alignment of multiple ITS and 5S rDNA sequences from all taxa was accomplished using the PILEUP module of the Wisconsin GCG computer package, version 8.0 (Devereux et al., 1984). After visual inspection of the resulting alignment, manual editing was done using MacClade 3.0. Boundaries of the coding and spacer sequences were determined by comparison with published ITS sequences from grass species (Hsiao et al., 1994) and 5S sequences from Hordeum L. sp (Baum and Johnson, 1996). Positions with ambiguous alignment were excluded from the analysis. All gaps were treated as missing data. The sequences reported in this study have been deposited with GenBank (Table 1).

Phylogenetic analysis

Phylogenetic analyses were performed using PAUP version 4.0b4a (Swofford, 1999). The program Modeltest, version 3.0 (Posada and Crandall, 2000), was used to find the model of sequence evolution which best fit each data set by the hierarchical likelihood ratio (LR) test (x = 0.01). Once the best sequence evolution model was determined [GTR for ITS regions (Yang et al., 1994) and K2P for 5S spacer (Kimura, 1980)], maximum likelihood (ML) tree searches were performed for each data set. Branch and Bound Search was used to find the maximum likelihood trees. Pairwise distance matrices calculated from the absolute distance between taxa and distance trees were obtained from neighbour-joining (NJ) analysis according to the model. Bootstrap values, taken as an index of support for individual clades, were calculated for 1000 replicates of the NJ analysis. The quartet puzzling method was used to test the confidence level of the ML trees with 1000 replicates.

RESULTS

Phylogenetic analysis—ITS region

All the double-stranded PCR products appeared as a sharp, single band on 1 % agarose gels with no evidence of different rDNA repeats. Complete sequences were obtained from all Clivia taxa and from the two outgroup species. The lengths of ITS regions were as follows: Clivia species (ITS1 248–250 bp, 5.8S 163 bp, ITS2 235 bp), H. coccineus (ITS1 245 bp, 5.8S 163 bp, ITS2 234 bp) and C. vanstonii (ITS1 243 bp, 5.8S 163 bp, ITS2 234 bp). The whole ITS1-5.8S-ITS2 region ranged from 640 to 648 bp.

Among Clivia taxa, pairwise sequence comparisons indicated sequence divergences (combined ITS1, 5.8S rDNA and ITS2) ranging from 0.46–2.52 %. The sequence divergence value for ITS1 and ITS2 ranged from 0.4–2.8 % and 0.9–3.4 %, respectively. The divergence values between Clivia taxa and outgroups varied from 10.91 to 15.51 %. When aligned, the sequences of the whole ITS regions yielded a matrix of 651 positions, of which 112 were variable (17.2 %). Of these, 53 were potentially phylogenetically informative (8.1 %). ITS1 contributed 11 of the informative characters, and ITS2 contributed 42 informative characters. Indels required 33 gaps for the correct alignment of sequences, and 30 of the gaps were due to the size difference between the ingroup and outgroup. Clivia nobilis had two fewer nucleotides than C. miniata, C. gardenii and ‘Robust Gardenii’ and one fewer than C. caulescens (Table 1).

One tree was generated using the maximum likelihood method with GTR model (-Ln likelihood = 1547.35; Fig. 2A). This tree grouped C. miniata, C. gardenii and ‘Robust Gardenii’ into one clade, C. gardenii and C. miniata were nested within this clade. This was strongly supported by the quartet puzzling test (91 and 100 % respectively). Clivia caulescens and C. nobilis formed a clade that is sister to the others (Fig. 2A), but this was not well supported by quartet puzzling test (57 %). The NJ tree using the same model shows that the clade of C. miniata, C. gardenii and ‘Robust Gardenii’ is the same as that of the ML tree (bootstrap values are 87 and 92 %). C. nobilis forms a clade on its own, distant from the three species. Clivia caulescens occupied an intermediate position between C. nobilis and the other three taxa (Fig. 2C).

Phylogenetic analysis—5S rDNA region

Incomplete 5S rDNA sequences were obtained when only one set of primers was used to amplify 5S rDNA repeats: two base pairs at positions 24 and 25 were absent when using 5SF and 5SR primers and 12 positions from 48 to 59 were absent when using SSP1 and SSP2 primers. Therefore, two different sets of primers were used to obtain overlapping sequences from the same region and complete sequences were obtained after comparing these sequences. On agarose gels the PCR products from C. miniata, C. gardenii, ‘Robust Gardenii’ and C. caulescens each produced a single band which, when cloned, was found to contain a uniform, species-specific sequence. In all cases, the sequence size of the whole region was 687 bp. The PCR product of C. nobilis produced two bands from which clones with three different insertions were obtained. These clones contained sequences that could be divided into two groups ['long' (856 bp) and ‘short’ (687 and 688 bp) sequences] due to differences in their spacer size. Two different sequences were obtained from L. aurea. They were 334 and 335 bp long.

The aligned sequences of the 5S rDNA repeat were 861 bp long. The coding regions were 120 bp long in both
ingroup and outgroup species and they were highly conserved. The spacers were considerably more variable, ranging from 567 to 736 bp between the different Clivia species and 215 bp in L. aurea. The sequence data showed that 5S rDNA spacer sequences were highly polymorphic in individual C. nobilis plants. Six clones were obtained from accession 8661 and four from 8659. These clones included three different sequences. Two of the sequences (seven clones) were similar in size to those in the other three species (567–568 bp), the other sequence was longer (736 bp; three clones). Seventy-three bp from the long sequence of C. nobilis were excluded because of ambiguous alignment in this region. The aligned spacer formed a 741 bp matrix for phylogenetic analysis. Among Clivia species, Kimura’s two-parameter pairwise (K2P) comparison showed that the sequence difference ranged from 0.18 to 9.36 %. The main sequence difference was between C. nobilis and the other taxa. Variation between the ingroup and outgroup ranged from 59.62 to 70.10 %. The aligned sequence showed 151 variable positions (57 %), of which 87 were potentially phylogenetically informative.

The phylogenetic tree generated using the maximum likelihood (ML) method with K2P is shown in Fig. 2B (-Ln = 1681.93). This tree is well resolved. The major topology grouped C. miniata, C. gardenii and ‘Robust Gardenii’ together, with C. miniata and C. gardenii forming a clade within this group. The quartet puzzling test strongly supported these clades (97 and 87 %, respectively). The long sequence and one short sequence from C. nobilis were grouped together, the other short sequence formed a sister clade to them. Clivia caulescens was placed between C. nobilis and the clade of the other three species. The support for most branches in the tree, after performing 1000 quartet puzzle steps, is high (over 80 %) (Fig. 2B). The tree produced using the NJ method with Kimura’s algorithm is similar to the ML tree (Fig. 2D).

Physical location of the 5S rRNA genes

There was only one pair of 5S rRNA gene sites in each of the four named Clivia species (Fig. 3A–D) and ‘Robust Gardenii’. Sequential Giemsa C-banding showed that these sites corresponded to the interstitial C-band on chromosome 8 in all species (Fig. 3E–H).

DISCUSSION

Sequence variation in the ITS and 5S rDNA of Clivia

No sequence information for either of these regions has been reported previously for the Amaryllidaceae. Kim and Jansen (1994) suggested that the two ITS regions were under different evolutionary constraints, but Baldwin et al. (1995) pointed out that in most plants pairwise divergence values were similar in both regions. This is supported by our results from Clivia. The sizes of ITS1 (248–250 bp) and
ITS2 (235 bp) are similar to those reported for other angiosperms (Baldwin et al., 1995; Kass and Wink, 1997; Andreasen et al., 1999). The sequence variation in Clivia is mainly due to point mutation. Although the physical location of the 45S rRNA genes using FISH indicated that there were either one, two or three pairs of hybridization sites in the different species (Ran et al., 1999), no intraspecific or inter-locus sequence variations were observed. This strongly suggests that concerted evolution of the ITS region occurs in Clivia as in many other plants (Baldwin et al., 1995).

Sequences of the 5S rRNA gene and spacer have been used for phylogenetic reconstruction in many plants (Steele et al., 1991; Kellogg and Appels, 1995; Udovicic et al., 1995; Baum and Johnson, 1996; Cronn et al., 1996; Crisp, 1999), but not previously in the Amaryllidaceae. The coding regions are relatively short with a low rate of base substitution and consequently provide few phylogenetically informative sites (Soltis and Soltis, 1998). In contrast, the spacer region has a much higher substitution rate and is phylogenetically more useful. However, alignment difficulties are likely to arise when more divergent taxa are involved due to the characteristic occurrence of simple repeats (Kanazin et al., 1993), indels (Cox et al., 1992) and the absence of inter-locus concerted evolution in 5S rDNA (Cronn et al., 1996). However, in other genera, strong concerted evolution in the 5S rRNA gene spacer has been found (Cronn et al., 1996). In Clivia, a single 5S rDNA sequence was obtained from each of C. miniata, C. gardenii ‘Robust Gardenii’ and C. caulescens, but in C. nobilis three different lengths and sequences were found. This variation was not reflected in the location of these sequences on the chromosomes as all species had only one pair of 5S rDNA sites all localized on chromosome 8. This contrasts with the situation in cereals where ‘long’ and ‘short’ sequences are characteristically located on different chromosomes (Scoles et al., 1988). The long sequence in C. nobilis has probably arisen from an insertion due to unequal exchange because almost all of the spacer regions of the short sequences could be aligned with the first part of the long sequence. The relatively high sequence variation and number of informative characters has shown that the 5S region provides sufficient useful information for phylogenetic analysis of Clivia.

In general, the sequence variation between Clivia taxa was low for both gene regions compared with that in other plant genera, such as Hypochoeris L. (Cerbah et al., 1998) and Allium L. subg. Melanocrommyum (Webb et Berth.) Rouy (Dubouzet and Shinoda, 1998) for the ITS region, and Hordeum (Sun et al., 1994) and Eucalyptus L’Herit. (Udovicic et al., 1995) for the 5S rDNA gene spacer. Except for the long sequence of 5S rDNA in C. nobilis, the divergence of the other sequences from the same gene in all these taxa was small.

Chromosome location of 5S rDNA gene sites

Given the variation in number and position of the 45S rDNA sites in Clivia (Ran et al., 1999) and the sequence variation found in the 5S rDNA in C. nobilis, it is interesting that the number and location of 5S sites is uniform in all the taxa. The more variable distribution of the 45S rDNA loci compared to that of the 5S rDNA in Clivia is paralleled in other genera such as Hordeum (Taketa et al., 1999), Picea A. Dietr. (Brown and Carlson, 1997) and Pinus L. (Jacobs et al., 2000). The 5S rRNA genes were located on the same chromosome as the 45S rRNA genes in four of the Clivia species, with C. gardenii being the exception. This is consistent with results from some plants (Mukai et al., 1990; Appels et al., 1992) but differs from others where the two sets of repeats are on different chromosomes (Ansari et al., 1999). Castilho and Heslop-Harrison (1995) and Taketa et al. (1999) have pointed out that the relative order of the 45S and 5S rRNA loci can help to identify chromosomes and clarify their structure and evolution. Our results suggest that chromosome 8 is highly conserved in all species of Clivia.

Phylogeny and chromosome evolution in Clivia

The phylogenies inferred from both the ITS regions and the 5S rDNA were similar. Both phylogenies suggest that C. miniata, C. gardenii and ‘Robust Gardenii’ are very closely related and this is reflected in their having very similar metaphase chromosome banding patterns, all chromosomes having centromeric bands (Fig. 4). The overlapping geographic distribution of these three species also suggests a rather recent divergence (Ran, 2000). In contrast, the phylogenies suggest that C. nobilis is basal to the other Clivia species, with C. caulescens located in an intermediate position between it and the other three species. A similar phylogenetic pattern was found when RAPDs were used to examine genetic relationships (Ran et al., 2001).

With a phylogeny it is now possible to suggest probable directions of karyotype evolution (Fig. 4). The three taxa with centromeric C-bands, C. miniata, C. gardenii and ‘Robust Gardenii’ are grouped together by the phylogenetic analysis and occupy the most derived position on the tree. The two most basal species on the tree, C. nobilis and C. caulescens, have no centromeric C-banding and it is therefore reasonable to conclude that the presence of centromeric C-bands is a derived condition. The species with centromeric C-bands were shown to have two-fold or greater amounts of heterochromatin than those without these bands (Ran et al., 1999).

The pattern of evolution of the 45S rDNA sites is slightly less clear-cut as there are two possible ancestral conditions (Fig. 4). The species have one, two or three pairs of the sites and they are distributed to chromosomes 2, 8, 9 or 10. In one scenario, the ancestral condition is that in which there are three sites on chromosomes 8, 9 and 10 as seen in the most basal species, C. nobilis. A loss of the site on chromosome 10 would result in an ancestor with the C. caulescens karyotype. The next step involves the translocation of the locus from the short arm of chromosome 9 to the long arm of chromosome 2. This has occurred in the ancestor of the three most derived species that cluster together, which are also characterized by the presence of centromeric C-bands. The final step appears to be the loss of the 45S site from chromosome 8 in C. gardenii: this
species is unique in having just a single pair of 45S sites. The alternative scenario involves the ancestral species having two pairs of 45S sites, on chromosomes 8 and 9, and the acquisition of an additional site on chromosome 10 in *C. nobilis*. At present it is not possible to determine the true ancestral condition as the two schemes are equally parsimonious. It is interesting that silver nitrate staining, which identifies 45S rDNA sites that were transcriptionally active in the preceding interphase, shows both pairs of sites to be active in *C. miniata* but one active pair in *C. caulescens* and *C. nobilis* (Ran et al., 1999). Unlike the 45S loci, the 5S rDNA locus in *Clivia* is found in the same position, associated with a C-band, on the same chromosome in all species.

**Classification of ‘Robust Gardenii’**

‘Robust Gardenii’ also known in South Africa as ‘Swamp Forest Gardenii’ (John Rourke, pers. comm.), was originally introduced to New Zealand as an accession of *C. gardenii*. However, on the basis of sequence variation obtained in this study, its unique pattern of karyotype markers (Fig. 4) and RAPD profile (Ran et al., 2001), it is clearly distinct from *C. gardenii*. A more detailed study of the morphology of this taxon is in progress so that its status and taxonomic placement can be determined and a formal description prepared.

**ACKNOWLEDGEMENTS**

We thank Allen Rodrigo for his help with the phylogenetic analyses, Karen Sheath for her help in the laboratory and Terry Hatch for supplying plants of *Cryptostephanus*.

**LITERATURE CITED**


