



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

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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 distantly 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. © 2001 Annals of Botany Company

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. All the named species are cross compatible and produce vigorous, fertile progeny (Hammett, unpubl. res.) suggesting a close relationship.

Recent studies on the karyotypes of the genus (Ran *et al.*, 1999) have confirmed 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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TABLE 1. *Species, source and GenBank accession numbers of the plants used in this study*

Species	Voucher	Source	ITS region		5S rDNA repeat	
			Size of repeat (bp)	Accession no.	Size of repeat (bp)	Accession no.
<i>C. caulescens</i>	K Hammett 880 (AKU)	Venus Plantation, South Africa (wild)	647	GBAN AF324021	687	GBAN AF324753
	K Hammett 881 (AKU)	Cultivated population, South Africa	647	GBAN AF324022	687	GBAN AF324754
<i>C. gardenii</i>	K Hammett 812 (AKU)	Cultivated population, via Japan	648	GBAN AF324023	687	GBAN AF324756
	K Hammett 827 (AKU)	South Africa, via UK (wild)	648	GBAN AF324024	687	GBAN AF324755
'Robust Gardenii'	K Hammett 882-1 (AKU)	Kirstenbosch, South Africa (wild)	648	GBAN AF324025	687	GBAN AF324757
	K Hammett 882-2 (AKU)	Kirstenbosch, South Africa (wild)	648	GBAN AF324026	687	GBAN AF324758
<i>C. miniata</i>	K Hammett 886 (AKU)	Howick, South Africa, (wild)	648	GBAN AF324027	687	GBAN AF324759
	K Hammett 8312 (AKU)	Walter's yellow clone, Italy via Australia (cultivated)	648	GBAN AF324028	687	GBAN AF324760
<i>C. nobilis</i>	K Hammett 8659 (AKU)	Kei River mouth, South Africa (wild)	646	GBAN AF324029	687 (short 1) 688 (short 2) 856 (long)	GBAN AF324761 GBAN AF324762 GBAN AF324763
	K Hammett 8661 (AKU)	Reit River, South Africa (wild)	646	GBAN AF324030	687 (short 1) 856 (long)	GBAN AF324764 GBAN AF324765
<i>Haemanthus coccineus</i>	K Hammett 98-1 (AKU)	Garden origin	642	GBAN AF324031		
<i>Cryptostephanus vansonii</i>	T Hatch 9801 (AKU)	Zimbabwe (wild)	640	GBAN AF324032		
<i>Lycoris aurea</i>	K Hammett 9801 (AKU)	Thai/Burma border (wild)				GBAN AF324766 GBAN AF324767

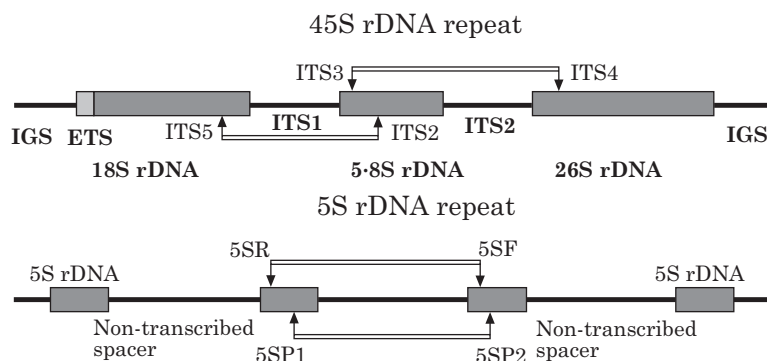


FIG. 1. Diagram showing the organization of 45S and 5S rDNA repeats and primer positions for PCR.

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 Phytpure kit (Amersham Pharmacia Biotech., Little Chalfont, Bucks, UK). The ITS1 and ITS2 regions

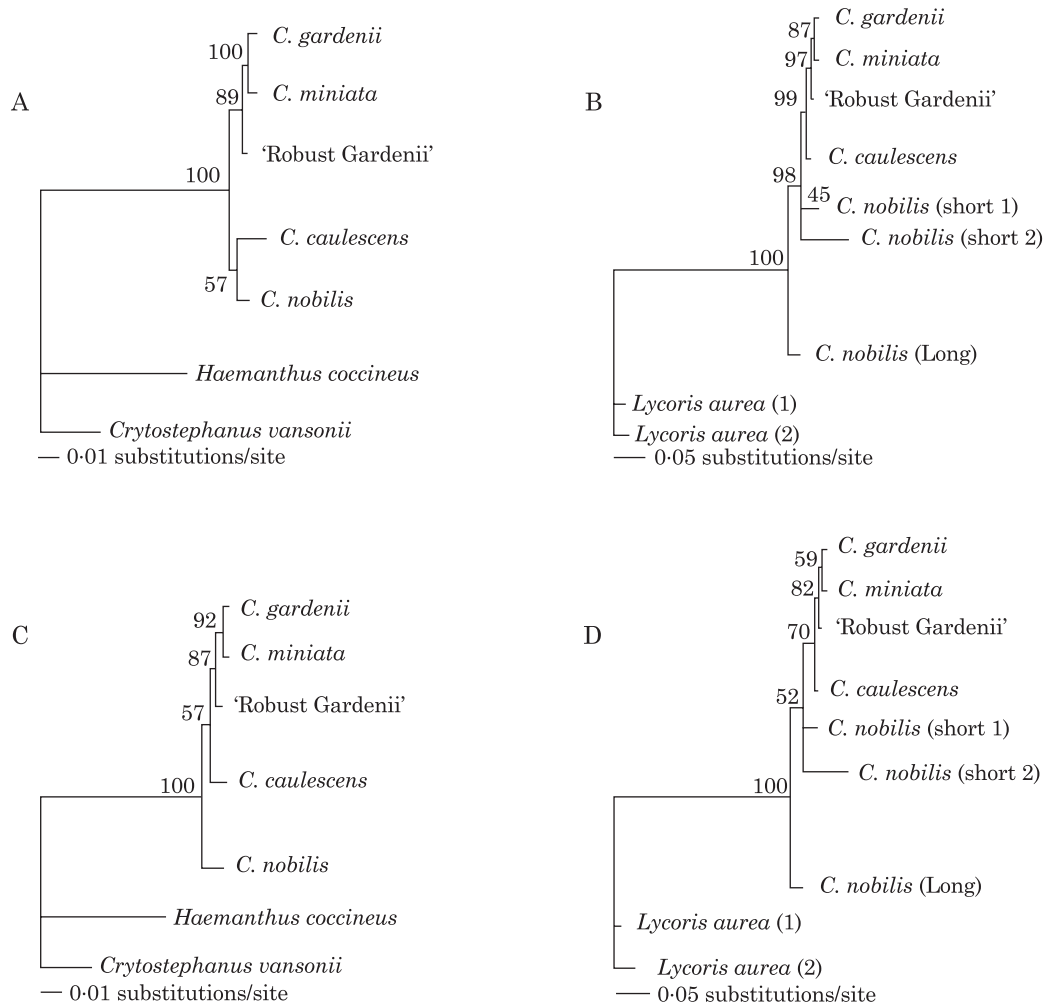


FIG. 2. Maximum likelihood trees generated from the entire ITS region (A) and 5S rDNA spacer (B) using GTR and K2P distance methods, respectively. The values above branches are percentages of how often the corresponding cluster was found among 1000 intermediate trees using the quartet puzzling test. 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.

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'-CACCGGATCCCATCA-GAACT-3') and 5SF (5'-TTAGTCTGGTATGATCGCA-3') (Udovicic *et al.*, 1995). The other set designed specifically for *Clivia* was 5SP1 (5'-GAGTTCTGATGG-GATCCGGTG-3') and 5SP2 (5'-TGCTTGGGCGAGAG-TAGTAC-3'). The positions of these primers on the 5S repeat are shown in Fig. 1). The PCR reaction was carried out in 25 μ l tubes containing 1 \times PCR reaction buffer, 0.2 mM dNTPs, 2 mM MgCl₂, 0.25 μ M 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. miniata*, *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 \times ligation buffer (Promega), followed by incubation at 4 $^{\circ}$ 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 $^{\circ}$ C for 1 h and then on LB agar plates containing 100 μ g ml⁻¹ ampicillin, 100 mM IPTG and 2% X-gal at 37 $^{\circ}$ 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 μ g ml⁻¹ ampicillin. Minipreparation of plasmid DNA was made with the Qiagen Minipreparation Kit, followed by

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 ($\alpha = 0.01$). 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. vansonii* (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 5SP1 and 5SP2 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

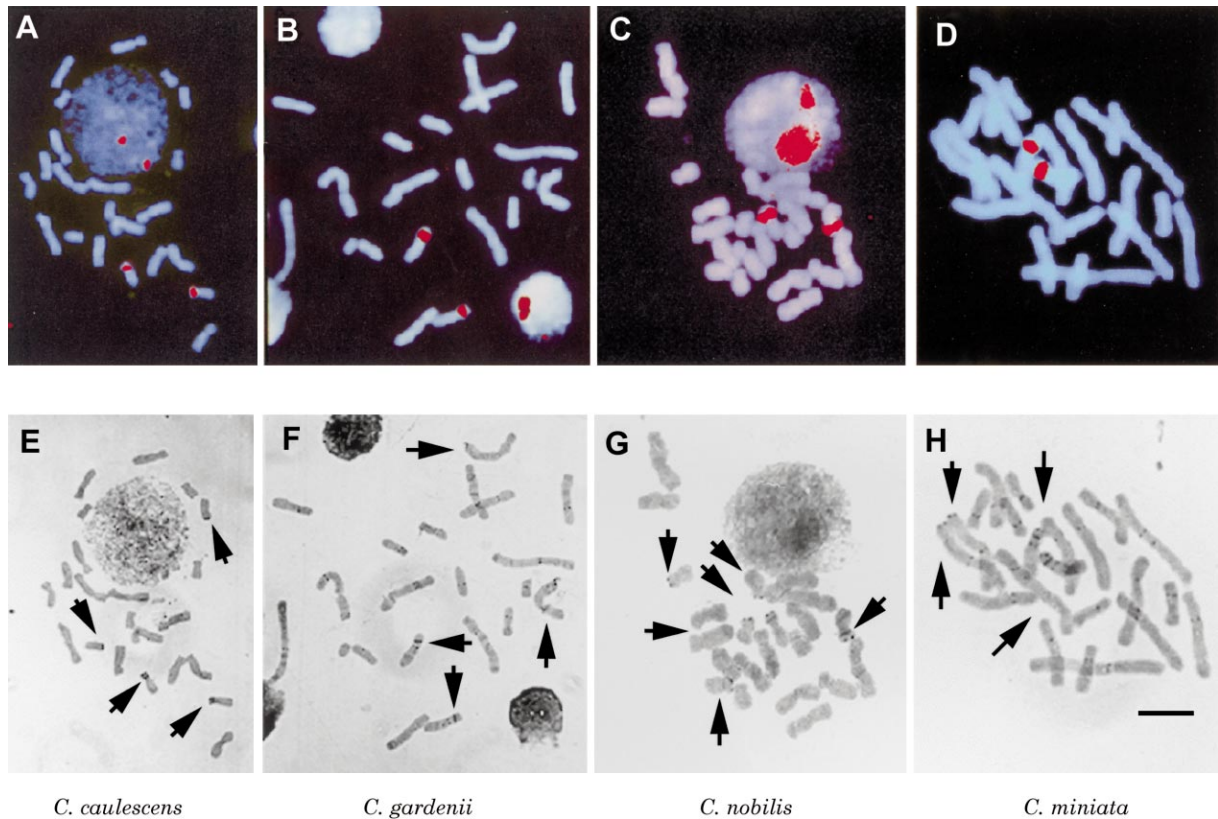


FIG. 3. *In situ* hybridization to mitotic chromosomes using the probe p55pr10 (5S rDNA). A, *C. caulescens*; B, *C. gardenii*; C, *C. nobilis*; D, *C. miniata*, and Giemsa C-banding on the same mitotic chromosome spreads of species of *Clivia*: E, *C. caulescens*; F, *C. gardenii*; G, *C. nobilis*; H, *C. miniata*. Arrows indicate informative chromosome variants. Bar = 10 μ m.

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; Andraesen *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 intra-specific 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 rRNA 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

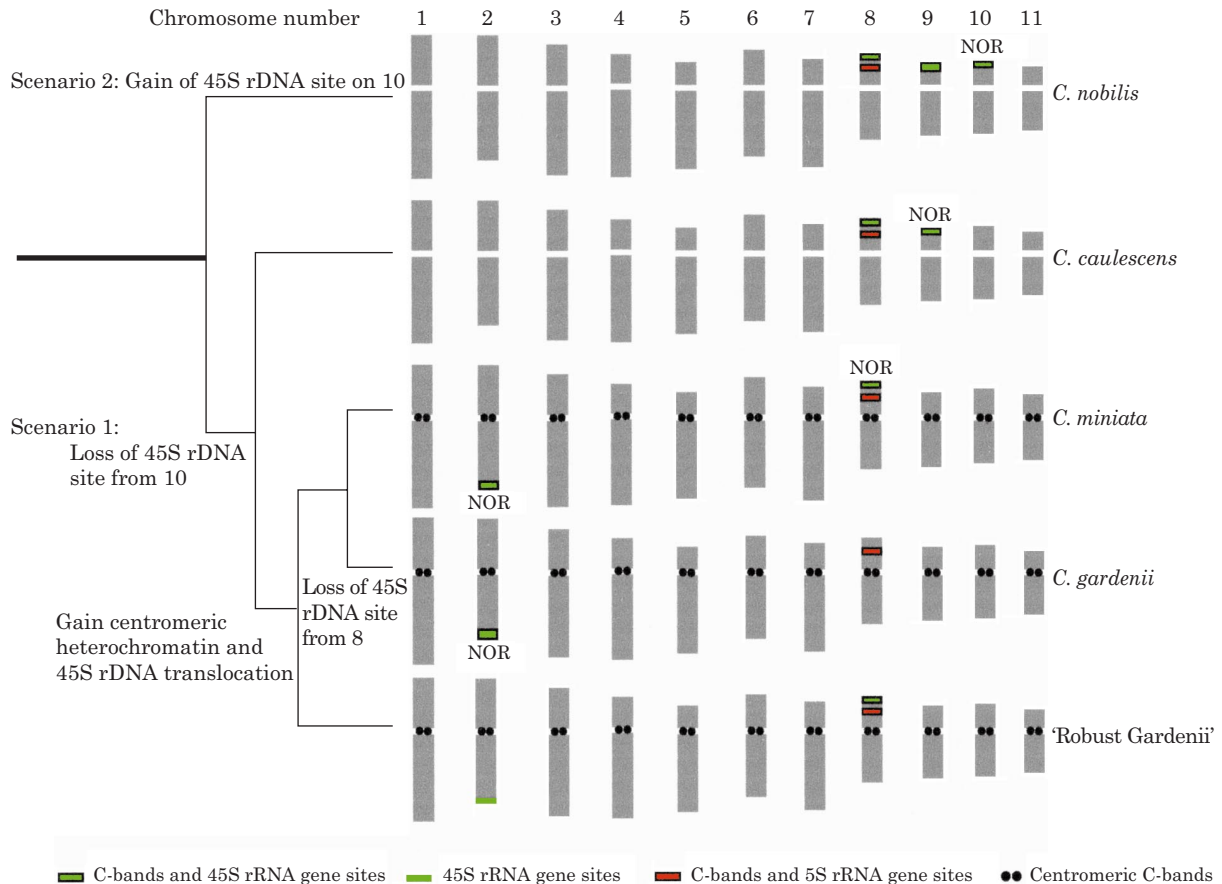


FIG. 4. Proposed phylogenetic relationships obtained from sequence data from the entire ITS region of the 45S rDNA and 5S rDNA spacer and chromosome evolution in *Clivia*. Two possible scenarios for the evolution of the 45S rDNA loci are presented as there are two equally parsimonious initial steps depending on whether the ancestral species had two or three loci. The number of active NORs in 'Robust Gardenii' is not presented as insufficient material was available for analysis.

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.

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