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ORIGINAL ARTICLE

The karyotype of *Clivia mirabilis* analyzed by differential banding and fluorescence in-situ hybridization

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Abstract The karyotype of the recently described species *Clivia mirabilis* was analyzed by differential chromosome staining with Giemsa, chromomycin, and DAPI and by fluorescence in-situ hybridization with 5S and 45S rDNA probes. Like the other five *Clivia* species it was shown to have a unique karyotype, although its karyotype was similar in several respects to that of *C. gardenii*, differing in having only one pair of chromosomes with CMA bands compared with two pairs in *C. gardenii* and lacking any DAPI-positive bands. The evolutionary relationships of the species and their karyotypes are discussed.

Keywords *Clivia mirabilis* · Chromosomes · Differential chromosome banding · FISH · Karyotypes

Introduction

Species and hybrids of *Clivia* are widely grown as ornamental plants in many parts of the world, either outdoors in shady situations or under glass (Koopowitz 2002). The genus contains six species, four of these, *C. miniata*, *C. gardenii*, *C. nobilis*, and *C. caulescens*, have been known for decades; more recently two new species, *C. mirabilis* and *C. robusta*, have been described (Rourke 2002; Murray et al. 2004). In an earlier study (Ran et al. 1999) we showed, using a combination of differential chromosome banding (Giemsa, DAPI, and chromomycin) and fluorescence in-situ hybridization (FISH), that in the *Clivia* species that had been described at that time, each species had a distinct karyotype. During that study we also observed that plants that had been identified by *Clivia* growers as a large or robust form of *C. gardenii* had a unique karyotype. This, together with a suite of morphological characters, led to the description of these plants, previously called 'Robust *gardenii*' or 'Swamp Forest *Clivia*', as *C. robusta* (Murray et al. 2004).

The phylogenetic relationships of the *Clivia* species have also been investigated using RAPD variation (Ran et al. 2001a), and nuclear (Ran et al. 2001b) and plastid (Conrad et al. 2003) DNA sequences. When their studies were done, Ran et al. (2001a, b) did not have access to material of *C. mirabilis* so their analyses was confined to the other five species whereas Conrad et al. (2003) did not include *C. robusta* in their study. Both studies found that *C. nobilis* was sister to a clade containing *C. miniata*, *C.gardenii*, and *C. caulescens*, although the relationships of these three species were slightly different in the two studies. Ran et al. (2001a, b) showed that *C. caulescens* was sister to *C. miniata* and *C. gardenii* whereas Conrad et al. (2003) found that *C. gardenii* was sister to *C. miniata* and *C. caulescens*. Ran et al. (2001b) used their phylogeny to infer the direction of evolutionary changes in karyotype with the suggestion that, for example, there had been a loss of 45S rDNA sites and addition of centromeric C-bands during karyotype evolution.

In this paper we investigate the karyotype of *C. mirabilis* using the same techniques as those used by Ran et al. (1999) and place these observations in a phylogenetic/evolutionary context.

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Materials and methods

Plant material

Two different accessions of *Clivia mirabilis* initially obtained from the Northern Cape Province, South Africa, but subsequently grown in Auckland, New Zealand, were used in this study. The exact place of origin of the plants is unknown but the species is confined to a restricted area of South Africa (Rourke 2002).

Chromosome preparation

Chromosome preparations were made from root-tip cells following the method of Murray and Davies (1996). Root tips were placed in a saturated solution of paradichlorobenzene at 4°C for 18–20 h, fixed in glacial acetic acid–absolute ethanol (1:3 *v*/*v*) for 24 h, then transferred to 70% ethanol and stored at −20°C until used. One or two root tips, depending on size, were cut into small pieces (c. 1 mm^2), placed in an Eppendorf tube, and washed twice with 0.1 M citrate buffer (pH 7) before being digested with a mixture of 4% cellulase (Onozuka R10; Merck) and 1% pectolyase (Sigma) in the same citrate buffer at 37°C for 30 min. During this time the material was aspirated with a Pasteur pipette at 5 min intervals. At the end of the digestion period the tube was centrifuged (6,000*g*), the enzyme mixture was removed, and the pellet was resuspended in citrate buffer, to remove the enzyme mixture, then centrifuged. This procedure was repeated before the buffer was removed and replaced with ten drops of 45% acetic acid. The cells were then resuspended in the acetic acid and one drop of the cell suspension was placed on a microscope slide, a cover slip was added, and the slide was heated and squashed to flatten and spread the cells. The cover slip was removed by immersing the slide in liquid nitrogen for c. 1 min. Slides were then left to dry overnight at 37°C and either stored at room temperature or processed as described below. Slides were stained with FLP orcein (Jackson 1973) to obtain solid stained chromosomes. Chromosome measurements were made on ten cells stained with orcein that had well-contracted (C-metaphase) chromosomes.

Giemsa C-banding

The C-banding procedure followed that of Ran et al. (1999), in which slides were first immersed in 45% acetic acid at 60°C for 10 min, then rinsed in deionized water for 10 min before being placed in a saturated solution of barium hydroxide at 25°C for 15 min. The barium hydroxide was then flushed out with deionized water, and the slides were dipped in 1% acetic acid for 1 min and washed with deionized water for 10 min before incubation in 2× SSC at 60°C for 90 min. They were then stained with 3% Giemsa (Sigma) for 15 min, rinsed in deionized water, and air-dried before mounting with DPX.

Chromomycin (CMA) banding

Slides were rehydrated in McIlvaine's buffer (pH 7) for 10 min at room temperature, then stained with 20 µl distamycin (0.2 mg/ml in McIlvaine's buffer + 5 mM $MgCl_2$ (pH 7)) in the dark for 15 min, rinsed for 2 min in McIlvaine's buffer + 5 mM $MgCl_2$ (pH 7), then stained with 25 µl chromomycin (0.5 mg/ml in McIlvaine's buffer + 5 mM $MgCl_2$ (pH 7)) for 120 min. They were then rinsed with McIlvaine's buffer + 5 mM $MgCl_2$, air dried, then mounted in 50% Citifluor + 50% McIlvaine's.

DAPI banding

Three different procedures were used to try to produce DAPI bands. The first involved treatment of air-dried slides with 0.15 M NaOH in 70% ethanol for 5–7 min followed by dehydration through an ethanol series (70, 85, and 100%) at −20°C. The slides were then air dried before mounting either in Vectashield containing DAPI (1.5 µg/ml, Vector Laboratories, USA) or in 0.3% DAPI in a solution of 0.2% DABCO and 50% glycerol in 1× PBS. A second method involved placing the slides in 45% acetic acid at 60°C for 10 min, rinsing in running water for 10 min, then placing the slides in a saturated solution of barium hydroxide at 24°C for 12 min. The slides were then dipped in 1% acetic acid for 1 min, washed with deionized water for 10 min, then air dried before staining with 20 µl DAPI (2µg/ml in McIlvaine's buffer (pH 7) for 2 min. Finally the slides were mounted with 0.2% DABCO and 50% glycerol in 1× PBS. The third method was a repeat of the second but the slides were stained sequentially with chromomycin for 40 min, distamycin for 15 min, then DAPI for 2 min with two rinses of 2 min with deionized water between treatments. Finally, slides were mounted in 50% DABCO in McIlvaine's buffer. Slides that were used for the FISH procedure were also counterstained with DAPI, as this procedure often results in DAPI banding.

Fluorescence in-situ hybridization (FISH)

The 5S and 45S rDNA probes were prepared by PCR, using forward and reverse primers for each locus and the genomic DNA of *Plantago raoulii* as the template. The 5S region was amplified using the primers S1 (5′-GGGTGC GATCATACCAGC-3′) and S2 (5′-GGGTGCAACACAA GGACTTCC-3′) (Shibata and Hizume 2002). The 45S region was amplified using CY1 (5′-TACCGATTGAAT

GATCCGGTGAAG-3′) and CY3 (5′-CGCCGTTACTAG GGGAATCCTTGT-3′) (Wright et al. 2006). The PCR products were labelled by nick translation with biotin-16-dUTP (5S) and digoxigenin-11-dUTP (45S), respectively (Roche, USA), following the manufacturer's instructions.

The FISH procedure followed Schwarzacher and Heslop-Harrison (2000). The slides and the hybridization mixture containing the probes in 40% formamide and 1× SSC were heated to 75°C for 5 min and the temperature was then reduced to 37°C for 18 h for hybridization to occur. A high-stringency post-hybridization wash containing 20% formamide and 0.1× SSC at 42°C was used so that sequences with <80–85% sequence similarity between the probe and the target were removed. After the post-hybridization washes, the slides were counter-stained with DAPI in Vectashield (Vector Laboratories, USA) and subsequently examined and photographed. The captured images were merged and adjusted for brightness and contrast using Adobe Photoshop CS4.

Results

Ran et al. (1999) provided a numbering scheme based on chromosome length and centromere location to identify the chromosomes of *Clivia* and that was followed here. *Clivia mirabilis* had the same chromosome number, $2n = 22$ and basic orcein karyotype as the five other *Clivia* species (Fig. 1a). The C-banded chromosomes have small, dot-like centromeric bands, a larger, more diffuse sub-terminal band on chromosome 8, and another terminal band on chromosome 2 (Fig. 1b). These bands appear to be DAPI negative but the C-band on chromosome 2 corresponds to the clear CMA band on chromosome 2 (Fig. 1c). FISH revealed a single 45S rDNA hybridization site at the end of chromosome 2 (Fig. 1d), in a similar position to the C and CMA bands on that chromosome. Similarly there was a single 5S rDNA hybridization site in the short arm of chromosome 8 (Fig. 1e) that was C-band positive and DAPI negative. The idiogram (Fig. 2) combines the locations of the C and CMA bands and the locations of the 5S and 45S rDNA hybridization sites.

Discussion

Previous studies (Ran et al. 1999; Murray et al. 2004) showed that each of the five species of *Clivia* had a unique karyotype with differences in the number and position of both chromosome bands and sites of in-situ hybridization to rDNA probes. *Clivia mirabilis* also has a unique karyotype. This shows some similarity to that of

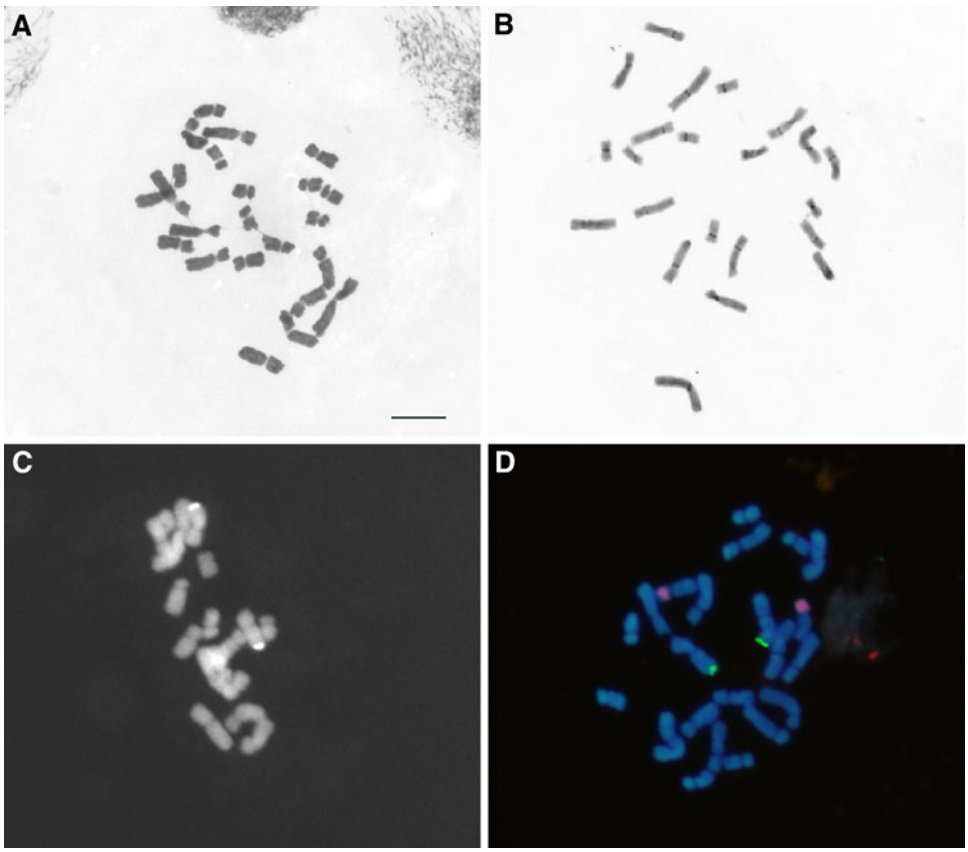

Fig. 1 Mitotic chromosomes of *Clivia mirabilis*. **a** Solid stained with FLP orcein. **b** Giemsa C-banded. **c** CMA banded. **d** FISH with the 45S rDNA probe (*green*) and the 5S rDNA probe (*red*). *Scale bar* 10 µm

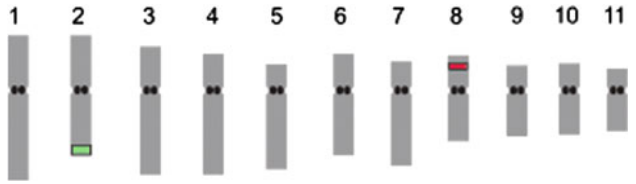

Fig. 2 Idiogram of the haploid chromosome complement of *Clivia mirabilis* showing the locations of the C and CMA bands and the locations of the 5S and 45S rDNA hybridization sites. *Green*, 45S rDNA site + CMA and C-band; *red*, 5S rDNA site + C-band; *black dots*, small centromeric C-band

C. gardenii, as they have the same number of rDNA hybridization sites at the same locations and their C-banding pattern is also similar. However, they differ in that *C. mirabilis* has only a single pair of CMA bands, on chromosome 2, whereas *C. gardenii* has two pairs, one on chromosome 2, in the same location to that of *C. mirabilis*, and the other on chromosome 8. The other clear difference is the absence of DAPI banding at the C-banded centromeres of *C. mirabilis.* All the other *Clivia* species that have centromeric C-bands, *C. miniata*, *C. gardenii*, and *C. robusta*, have DAPI bands at the corresponding positions to the C-bands. It is always difficult to prove a negative result, here the absence of DAPI banding in *C. mirabilis*, but use of several different DAPI staining schedules and observing the chromosomes that were used for the FISH schedule that were counterstained with DAPI all gave the same negative result. C-bands that do not show differential staining with CMA or DAPI have been found in other plant groups, for example the work of Berg and Greilhuber (1993a, b) in *Cestrum* species.

The phylogenetic tree produced by Conrad et al. (2003) places *C. mirabilis* as basal to all *Clivia* species. Zonneveld (2002) has also suggested on the basis of genome size measurements that *C. mirabilis*, with the smallest DNA C-value for the genus, is basal to the other species. Ran et al. (2001a, b) did not have access to material of *C. mirabilis* when they produced their phylogeny but when they mapped the karyotypic characters of the species on to their tree they found that the two most basal species, *C. nobilis* and *C. caulescens*, lacked the centromeric C-bands present in the other species. They suggested that an absence of C-bands was the ancestral state but if *C. mirabilis* is basal to all other species of the genus, as suggested by Conrad et al. (2003), this cannot be true and, perhaps, the processes of karyotypic change in *Clivia* are more complex than originally proposed by Ran et al. (2001b). There does not seem to be any simple resolution of this but there is now ample evidence for both increases and decreases in C-value and for the loss and gain of chromosome bands and in-situ hybridization sites associated with evolution of the genomes of several angiosperm genera (Murray 2002; Lysak et al. 2009). Irrespective of the direction of karyotypic change it is clear that *C. mirabilis* is, like the other species of *Clivia*, characterized by a unique karyotype.

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