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# Comparative molecular cytogenetics of major repetitive sequence families of three *Dendrobium* species (Orchidaceae) from Bangladesh

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• *Background and Aims Dendrobium* species show tremendous morphological diversity and have broad geographical distribution. As repetitive sequence analysis is a useful tool to investigate the evolution of chromosomes and genomes, the aim of the present study was the characterization of repetitive sequences from *Dendrobium moschatum* for comparative molecular and cytogenetic studies in the related species *Dendrobium aphyllum*, *Dendrobium aggregatum* and representatives from other orchid genera.
• *Methods* In order to isolate highly repetitive sequences, a $c_0t$*-1* DNA plasmid library was established. Repeats were sequenced and used as probes for Southern hybridization. Sequence divergence was analysed using bioinformatic tools. Repetitive sequences were localized along orchid chromosomes by fluorescence *in situ* hybridization (FISH).
• *Key Results* Characterization of the $c_0t$*-1* library resulted in the detection of repetitive sequences including the $(GA)_n$ dinucleotide DmoO11, numerous *Arabidopsis*-like telomeric repeats and the highly amplified dispersed repeat DmoF14. The DmoF14 repeat is conserved in six *Dendrobium* species but diversified in representative species of three other orchid genera. FISH analyses showed the genome-wide distribution of DmoF14 in *D. moschatum*, *D. aphyllum* and *D. aggregatum*. Hybridization with the telomeric repeats demonstrated *Arabidopsis*-like telomeres at the chromosome ends of *Dendrobium* species. However, FISH using the telomeric probe revealed two pairs of chromosomes with strong intercalary signals in *D. aphyllum*. FISH showed the terminal position of 5S and 18S–5·8S–25S rRNA genes and a characteristic number of rDNA sites in the three *Dendrobium* species.
• *Conclusions* The repeated sequences isolated from *D. moschatum* $c_0t$*-1* DNA constitute major DNA families of the *D. moschatum*, *D. aphyllum* and *D. aggregatum* genomes with DmoF14 representing an ancient component of orchid genomes. Large intercalary telomere-like arrays suggest chromosomal rearrangements in *D. aphyllum* while the number and localization of rRNA genes as well as the species-specific distribution pattern of an abundant microsatellite reflect the genomic diversity of the three *Dendrobium* species.

**Key words:** Orchidaceae, *Dendrobium moschatum*, *Dendrobium aphyllum*, *Dendrobium aggregatum*, repetitive DNA, FISH, $c_0t$*-1* DNA.

## INTRODUCTION

Orchidaceae is the largest family among angiosperms, consisting of about 850 genera with 25 000 species (Dressler, 1993). *Dendrobium* is the third largest genus of Orchidaceae comprising, at the time of writing, of 1184 species (Leitch *et al.*, 2009). *Dendrobium* species are characterized by a broad geographical distribution, a tremendous diversity in growth habits and the ability to produce a large number of interspecific hybrids with different morphology. The systematics of the subtribe Dendrobiinae was extensively studied on the basis of morphological key characters (Dressler, 1981), and on the basis of chloroplast DNA sequences (Yukawa *et al.*, 1996, 2000). However, the classification of many *Dendrobium* species remains ambiguous (Clements, 2003).

For Bangladesh, 16 *Dendrobium* species have been described (Hossain, 2002), some of which either have economical value as ornamental plants or are regarded as endangered species. In cytogenetic analyses, karyotypes of some *Dendrobium* species from Bangladesh have been compared (Begum and Alam, 2004, 2005). However, unequivocal species differentiations are hampered by almost similar chromosome numbers ($2n = 38–40$) and only few differences in chromosome morphology.

Repetitive sequence families are major components of plant genomes (Heslop-Harrison, 2000). According to genomic organization, repeats are divided into tandemly arranged and dispersed sequences (Schmidt and Heslop-Harrison, 1998). Tandem repeats are divided into satellite DNA, micro- and minisatellites, telomeric repeats and ribosomal genes. Typical plant satellite DNA repeats range in size between 160–180 and 320–360 bp and are organized in tandem arrays with up to $10^5$ copies per haploid genome (Hemleben *et al.*, 2000). Microsatellites are simple sequence repeats representing a unique type of tandemly repeated genomic sequences. They are abundantly distributed in small arrays across the genome and show high levels of polymorphism in sequence and copy number. Some tandem repeats are also functional. The ends of eukaryotic chromosomes form a unique chromatin domain that comprises the telomeres

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and the adjacent subtelomeric regions, often consisting of degenerated telomere sequences. In most plants, telomeres are tandem arrays of the repeat unit 5′-TTTAGGG-3′, which is widely distributed among the majority of plant species (Richards and Ausubel, 1988; Ganal *et al.*, 1991). The most conserved tandemly arranged sequences are ribosomal RNA genes in eukaryotes comprising 18S–5·8S–25S rRNA repeating units forming long arrays.

The rapid evolution of both tandemly arranged and dispersed repetitive DNA often results in species-specific repeat variants and the generation of novel sequence families. Therefore, comparative studies of plant repetitive sequences are useful to investigate the evolutionary relationships between plant species (Kamm *et al.*, 1995) and suggest that comparative study of repetitive sequences would be useful for investigation of the relationships among orchid species.

The present paper describes the molecular characterization of repetitive sequences including dispersed, telomeric and microsatellite repeats in three *Dendrobium* species. The genomic organization of repetitive DNA sequences was elucidated by comparative Southern hybridization in *Dendrobium* species and species of distantly related orchid genera. Physical mapping by fluorescence *in situ* hybridization (FISH) was used to show the distribution of the different types of repeat families as well as 18S–5·8S–25S and 5S rRNA genes along chromosomes.

## MATERIALS AND METHODS

### *Plant material and genomic DNA extraction*

Nine different orchid species were used, six of which belong to the genus *Dendrobium* and the remaining three to different genera. *Dendrobium moschatum* Sw., *Dendrobium aphyllum* Roxb. and *Dendrobium aggregatum* Roxb. were collected in Bangladesh. *Dendrobium anosmum* L., *Dendrobium fimbriatum* var. *oculatum* Hook.f., *Dendrobium palpebrae* L. and other orchid species *Nageliella angustifolia* (Booth ex Lindl.) Ames et Correl., *Oncidium sphacelatum* L. and *Phalaenopsis lueddemanniana* var. *purpurea* (Rchb.f.) Sweet. were obtained from the Dresden Botanical Garden, Germany.

Genomic DNA from each species was isolated from young leaves using the DNeasy Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

### *Chromosome preparation*

Primary roots were collected and washed in running tap water for 4–5 min. After removal of excess water, roots were incubated in 8-hydroxyquinoline (0·002 M) for 5 h at 18 °C, fixed in 45 % acetic acid for 15 min at 4 °C and stored in 70 % ethanol at 4 °C. Fixed roots were washed in enzyme buffer (0·01 M citric acid–sodium citrate, pH 4·6) to remove the fixative and digested at 37 °C for 2 h in enzyme solution consisting of 2·5 % pectinase, 2·5 % cellulose, 2·5 % pectolyase and 1·0 % cytohelicase in enzyme buffer. The dropping method was applied for the preparation of mitotic metaphase chromosomes according to Schwarzacher and Heslop-Harrison (2000).

### *Fluorescence* in situ *hybridization*

FISH probes were labelled with biotin-16-dUTP or digoxigenin-11-dUTP by PCR or nick translation, respectively. Sites of hybridization were detected immunologically by antibodies coupled to fluorochromes. The clone pTa71 from *Triticum aestivum* (Gerlach and Bedbrook, 1979) consisting of a large part of the 18S–5·8S–25S rRNA genes was labelled with digoxigenin-11-dUTP by nick translation, while the clone pXV1 (Schmidt *et al.*, 1994) containing the 5S rRNA gene from *Beta vulgaris* was labelled with biotin-16-dUTP using PCR. Probes of *D. moschatum* were labelled with biotin-16-dUTP (clone DmoF14) and digoxigenin-11-dUTP (clone DmoO11). The telomeric sequence (clone DmoB22) was labelled with digoxigenin-11-dUTP using PCR.

FISH was performed according to Heslop-Harrison *et al.* (1991). Chromosome spreads were pre-treated with 100 μg $mL^{-1}$ RNase A in 2× sodium saline citrate (SSC) for 1 h at 37 °C and washed twice in 2× SSC. After incubation with 10 μg $mL^{-1}$ pepsin in 0·01 mM HCl for 20 min at 37 °C, preparations were stabilized in freshly de-polymerized 4 % (w/v) paraformaldehyde in water for 10 min, dehydrated in a graded ethanol series and air dried. The hybridization mixture, consisting of 50–150 ng $\mu L^{-1}$ of DNA probe, 50 % (v/v) formamide, 10 % (w/v) dextran sulfate, 0·1 % sodium dodecyl sulfate (SDS) and 300 ng $\mu L^{-1}$ of sheared salmon sperm DNA in 2× SSC, was incubated for 10 min at 70 °C and chilled on ice. Then, 30 μL of the hybridization mixture was added to the chromosome preparations and covered with a plastic coverslip. The hybridization mixture and the chromosomal DNA were denatured at 70 °C for 5 min and the temperature was gradually decreased to 37 °C using a Hybaid Omnislide temperature cycler (Thermo, Waltham, MA, USA). Hybridization was carried out overnight at 37 °C. Following hybridization, the slides were given a stringent wash in 20 % (v/v) formamide in 0·1× SSC at 42 °C to remove mismatched or unhybridized probe molecules. For the detection of digoxigenin- or biotin-labelled probes, slides were equilibrated in 4× SSC/0·1 % (v/v) Tween 20 and blocked in 5 % (w/v) bovine serum albumin in 4× SSC/0·1 % (v/v) Tween 20 for 5 min. Slides were incubated with a final concentration of 2 μg $mL^{-1}$ of sheep anti-digoxigenin antibody conjugated with fluorescein isothiocyanate (FITC) or streptavidin-Cy3 in a moist chamber at 37 °C for 1 h. Excess antibody was removed by washing the slides in 4× SSC/0·1 % (v/v) Tween 20 three times for 5 min. After counterstaining with DAPI (4′,6-diamidino-2-phenylindole; 2 μg $mL^{-1}$), the slides were mounted in antifade solution (AF1, Citifluor). Examination of slides was carried out with a Zeiss Axioplan2 fluorescence microscope equipped with filters 09 (FITC), 15 (Cy3) and 01 (DAPI). Images were acquired directly with Applied Spectral Imaging v. 3·3 software, coupled with a high-resolution ASI BV300-20A CCD camera, and printed from Adobe Photoshop after contrast optimization using only functions affecting the whole image equally.

### *Construction of a* $c_0$t-1 *DNA library*

The *$c_0$t-1* DNA for isolation of repetitive sequences with high or moderate copy number was prepared according to the protocol from Zwick *et al.* (1997) with minor

modifications. Genomic DNA (100 ng $\mu L^{-1}$) from *D. moschatum* was sheared at 99 °C for 10 min followed by sonication at 80 °C for 3 min to generate fragments ranging in size predominantly between 0·5 and 1·0 kb. Renaturation of DNA fragments was carried out in a 0·3 M NaCl solution at 65 °C after initial denaturation at 92 °C for 10 min. The renaturation time was calculated according to Zwick *et al.* (1997). Following S1 nuclease (Promega, Madison, WI, USA) treatment to remove single-stranded DNA and single-strand overhangs on renaturated double-stranded DNA, enzyme inactivation was carried out with stop solution [3 M Tris, pH 8·0, 0·5 M ethylenediaminetetraacetic acid (EDTA)] according to Ostermeier *et al.* (1999) and incubation at 72 °C for 20 min. Blunt-ended *$c_0$t-1* DNA fragments were used for ligation into the *Sma*I site of the dephosphorylated pUC18 vector. After transformation in XL1Blue cells (Stratagene, La Jolla, CA, USA) by electroporation, positive clones were transferred to 384-well plates, grown in LB freezing medium and stored at −80 °C. For the identification of repetitive DNA sequences, clones were spotted onto nylon membranes (GE Healthcare, Chalfont, UK) and sequentially probed with radiolabelled genomic DNA of *D. moschatum*, *D. aphyllum* and *D. aggregatum*. The overnight hybridizations were performed at 60 °C in 5× SSPE (0·75 M NaCl, 50 mM $NaH_2PO_4$, 5 mM EDTA, pH 7.0) with 5× Denhardt's solution and 0·2 % SDS. Post-hybridization washings were performed twice at 60 °C in 1× SSC/0·1 % SDS for 10 min. The signals were detected by autoradiography.

### *PCR amplification of DmoF14 repeats*

For amplification of the dispersed repeat DmoF14 from different orchid species, the primer pair 5′-CAGTTCACAAA GAGCTAATGC-3′ and 5′-TAGTCAGAGGTAGTCGACCC AACC-3′ was used for PCR with genomic DNA as template at an annealing temperature of 54 °C. PCR reactions with 50 ng template DNA and a final primer concentration of 0·5 μM were performed in a volume of 50 μL containing 0·2 mM dNTPs, 50 mM KCl, 1·5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9·0) and 1 unit of Taq DNA polymerase (GE Healthcare). Standard PCR conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 49–55 °C for 45 s and 72 °C for 3·5 min, and a final incubation at 72 °C for 10 min.

After gel electrophoresis, PCR fragments were purified with the QIAquick gel extraction kit (Qiagen) and cloned in the pGEM-T vector (Stratagene).

### *Sequence analyses*

Plasmid clones from the *$c_0$t-1* DNA library and plasmid clones harbouring the dispersed repeat DmoF14 amplified from different orchid species were sequenced on a CEQ 8000 capillary sequencer (Beckman, Fullerton, CA, USA). Sequences from the *$c_0$t-1* DNA library were aligned via the MegAlign option within the Lasergene 6·0 software (DNAStar, Madison, WI, USA) by using CLUSTAL with default parameters. Divergence between DmoF14 in different orchid species was analysed by the neighbour-joining algorithm of the MEGA4·0 program (Tamura *et al.*, 2007).

### *Southern hybridization*

Southern hybridization to genomic DNA of different orchid species was performed using standard conditions with $^{32}$P-labelled probes (Sambrook *et al.*, 1989). DNA from all nine species used was digested with different restriction endonucleases, separated on 1·1 % agarose gels and transferred to HybondN+ (GE Healthcare) membranes. After hybridization, filters were washed at 60 °C in 2× SSC/0·1 % SDS and 1× SSC/0·1 % SDS for 10 min each. Signals were detected by autoradiography.

## RESULTS

### *Identification of repetitive DNA sequences in* D. moschatum

To identify repetitive DNA sequences in *Dendrobium* species, the restriction endonucleases *Bam*HI, *Xba*I, *Hin*dIII, *Alu*I, *Rsa*I, *Hae*III, *Pst*I, *Sac*I, *Sma*I and *Kpn*I were used to digest total genomic DNA of *D. moschatum*. After gel electrophoresis, distinct bands of highly abundant repetitive DNA families were not observed. Therefore, as an alternative approach, a *$c_0$t-1* plasmid library from *D. moschatum* was constructed.

Experimental parameters were chosen to enrich repetitive sequences ranging from 100 to 1000 bp. The *$c_0$t-1* plasmid library consists of 384 clones which were separately hybridized with genomic DNA of *D. moschatum*, *D. aphyllum* and *D. aggregatum*. Based on the strength of the signals, eight clones harbouring highly repetitive DNA sequences were identified and designated Dmo with the corresponding plate coordinates.

Sequence analysis of these eight clones revealed six sequences ranging in size from 87 bp (DmoB22, EMBL accession no. FM212245) to 150 bp (DmoB8, DmoG14, DmoH14, DmoK14 and DmoO19) consisting of variable numbers of the *Arabidopsis*-like 5′-TTTAGGG-3′ telomeric sequences. The strong conservation without any degeneracy of the telomeric repeat motifs indicates their origin from the physical ends of the *D. moschatum* chromosomes.

The clone DmoO11 (EMBL accession no. FM212246) was identified as a microsatellite array of 30 copies of the dinucleotide GA. The clone DmoF14 (EMBL accession no. FM212247) has a length of 420 bp and showed no significant homologies with other sequences in the EMBL/GenBank database.

### *Genomic organization of repetitive sequences in* Dendrobium *species*

Southern hybridization was performed to investigate the genomic organization and abundance of the repeats isolated from the *D. moschatum $c_0$t-1* library. DmoB22 as a representative clone containing conserved telomeric repeats was hybridized to genomic DNA of *D. moschatum.* The hybrization signals extended over a wide range of fragments detectable as a smear over all lanes up to 20 kb (data not shown). Conserved bands originating from proximal degenerated repeats were not detected.

For analysis of the genomic organization of the DmoO11 and DmoF14 repeats, Southern analyses were performed

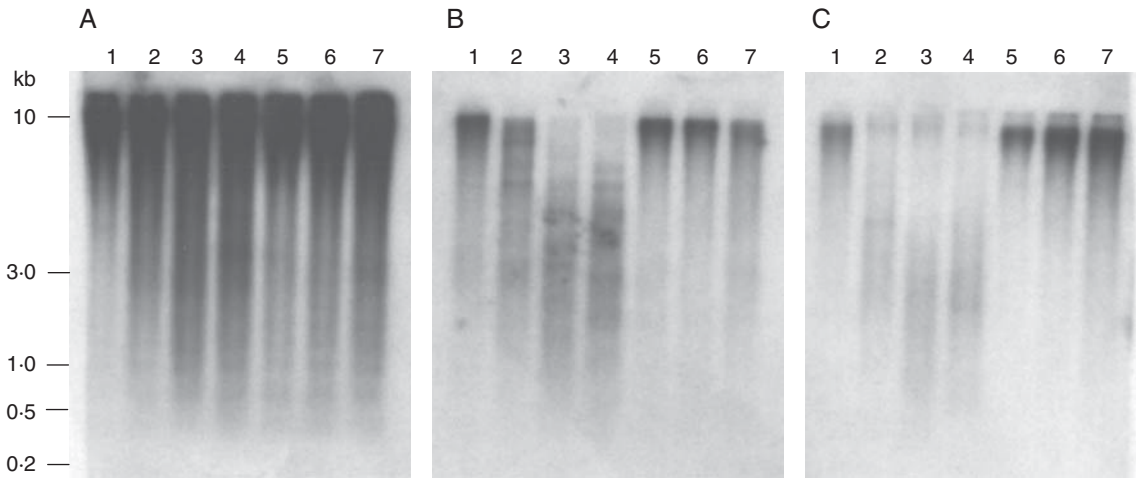


FIG. 1. Comparative Southern hybridization of the $(GA)_{30}$ microsatellite DmoO11 with genomic DNA of *D. moschatum* (A), *D. aphyllum* (B) and *D. aggregatum* (C), digested with *Bam*HI (lane 1), *Hae*II (lane 2), *Hin*fI (lane 3), *Rsa*I (lane 4), *Pst*I (lane 5), *Hpa*II (lane 6) and *Msp*I (lane 7). Molecular size markers are given on the left.

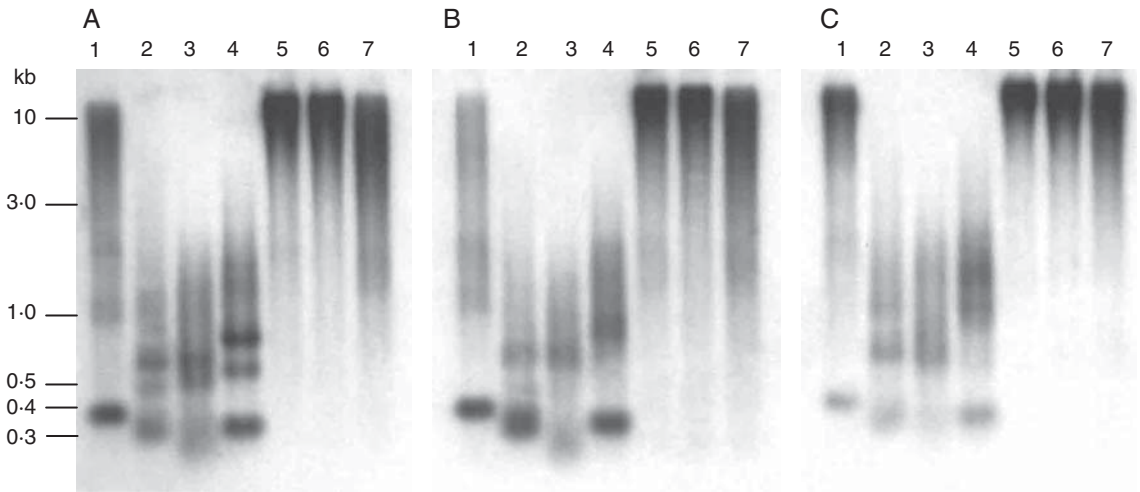


FIG. 2. Comparative Southern hybridization of the dispersed repeat DmoF14 with genomic DNA of *D. moschatum* (A), *D. aphyllum* (B) and *D. aggregatum* (C), digested with *Bam*HI (lane 1), *Hae*II (lane 2), *Hin*fI (lane 3), *Rsa*I (lane 4), *Pst*I (lane 5), *Hpa*II (lane 6) and *Msp*I (lane 7). Molecular size markers are given on the left.

with genomic DNA of *D. moschatum*, *D. aphyllum* and *D. aggregatum.*

In *D. moschatum*, the $(GA)_n$ microsatellite DmoO11 showed hybridization signals of strong intensity over the whole range of separation, ranging from 3 to 10 kb (Fig. 1A). In contrast, the intensity of the hybridization in *D. aphyllum* and *D. aggregatum* was significantly weaker (e.g. *Hin*fI and *Rsa*I), indicating a lower abundance of the $(GA)_n$ microsatellite in these two species compared with *D. moschatum* (Fig. 1B and C). Moreover, *D. aphyllum* genomic DNA restricted with *Hin*fI and *Rsa*I (Fig. 1B, lanes 3 and 4) revealed hybridization signals only in the range 1·0 kb to approx. 5·0 kb and reduced signals with larger genomic fragments. A similar but weaker hybridization with smaller fragments was also observed in *D. aggregatum* (Fig. 1C, lanes 3 and 4).

Hybridization of DmoF14 to genomic DNA of *D. moschatum*, *D. aphyllum* and *D. aggregatum*, each digested with *Bam*HI, *Hae*III, *Hin*fI and *Rsa*I, revealed strong signals detectable as conserved bands of different size and a smear over the whole lane, suggesting a dispersed organization of the DmoF14 repeat family (Fig. 2A–C). Of note, a prominent *Bam*HI fragment of approximately 375 bp corresponding to the distance between two *Bam*HI sites in DmoF14 was observed in *D. moschatum* and *D. aphyllum* (Fig. 2A, B, lane 1). A similar but slightly weaker pattern was observed in *D. aggregatum* (Fig. 2C, lane 1).

In *D. moschatum* the *Rsa*I restriction revealed three strong fragments between 400 and 750 bp (Fig. 2A, lane 4). The 400-bp fragment was conserved in *D. aphyllum* whereas it was weaker in *D. aggregatum* (Fig. 2B, lane 4).

Distinct *Hae*III and *Hin*fI fragments ranging from approx. 400 to 700 bp were localized in all three *Dendrobium* species (Fig. 2A–C, lanes 2 and 3). The methylation-sensitive enzymes *Hpa*II and *Msp*I showed strong hybridization in

a high-molecular-weight range (Fig. 2A–C, lanes 6 and 7). Similarly, the large *Pst*I fragments indicated strong methylation of the DmoF14 repeat.

### *Divergence of the dispersed repeat DmoF14 in orchid species*

As the Southern hybridization patterns of DmoF14 are highly conserved in the *Dendrobium* species analysed, other *Dendrobium* species and species from different orchid genera were included in comparative Southern hybridization experiments to elucidate whether DmoF14 is present in closely and distantly related species.

Therefore, genomic DNA of *D. moschatum*, *D. anosmum*, *D. fimbriatum* var. *oculatum* and *D. palpebrae* and species of different orchid genera (*Nageliella angustifolia*, *Oncidium sphacelatum* and *Phalaenopsis lueddemanniana* var. *purpurea*) was digested with *Bam*HI and *Rsa*I and hybridized with DmoF14. In both restriction enzymes, strong fragments of approx. 400 and 320 bp, respectively, were visible in all *Dendrobium* species (Fig. 3A–D, lanes 1 and 2). However, the strength of hybridization signals in *D. palpebrae* was considerably weaker (Fig. 3D).

In contrast, a smear without conserved fragments was observed in *Nageliella angustifolia* and *Oncidium sphacelatum* (Fig. 3E, F) whereas in *Phalaenopsis lueddemanniana* var. *purpurea*, very faint hybridization was detected only after longer exposure (Fig. 3G).

To investigate further the divergence of the DmoF14 repeat family in the orchid species studied and to complement the Southern hybridization results, repeat-specific primers were designed. PCR products were cloned and three independent clones from each species were sequenced (EMBL accession nos FM212248–FM212273). In order to display the structural relatedness of the species-specific DmoF14 sequences, a dendrogram was generated by a ClustalW alignment followed by a neighbour-joining analysis (Fig. 4). The tree showed two major clades supported by a 100 % bootstrap value. All *Dendrobium* DmoF14 repeats were grouped in one clade. The corresponding low bootstrap values indicate that many sequences analysed form a DmoF14 subfamily that is widespread in species of the genus *Dendrobium*. A second, more diverged subfamily consists of the *Dendrobium* repeats Dpa 1, Dfi 3, Dmo 3, Dan 1, Dag 2 and Dag 3.

The species of the three other orchid genera were separately grouped in distinct genus-specific clades, as supported by the corresponding high bootstrap values. The overall dendrogram revealed that the DmoF14 sequence is considerably diverged in species outside the genus *Dendrobium*. The divergence among *Dendrobium* species ranged from 2·3 to 10 %. The highest divergence (32–34 %) was observed between *Dendrobium* species and *P. lueddemanniana* var. *purpurea.* Of particular note is a single 35–38-bp deletion in *N. angustifolia*, which did not strongly affect the position of the DmoF14 sequences of this species in the dendrogram.

### *Physical mapping of repetitive sequences along orchid chromosomes*

FISH has been applied to physically map the identified repeats along chromosomes of *Dendrobium* species and to reveal aspects of chromosome evolution. As the chromosomes of orchid species are small and numerous and plant material is limited, cytogenetic analyses have to be performed with great care.

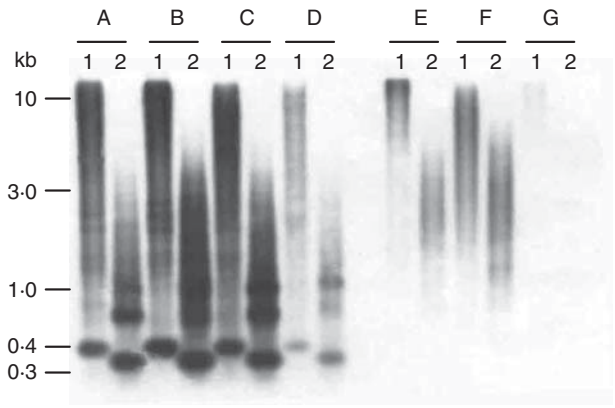


FIG. 3. Analysis of species distribution of DmoF14 by comparative Southern hybridization *of D. moschatum* (A), *D. anosmum* (B), *D. fimbriatum* var. *oculatum* (C), *D. palpebrae* (D), *N. angustifolia* (E), *O. sphacelatum* (F) and *P. lueddemanniana* var. *purpurea* (G). The DNA of each species was digested with *Bam*HI (lane 1) and *Rsa*I (lane 2). Molecular size markers are given on the left.

To compare the chromosomal distribution pattern of the three isolated *Dendrobium* repeats, FISH was performed on metaphase chromosomes of *D. moschatum* ($2n = 2x = 40$), *D. aphyllum* ($2n = 2x = 38$) and *D. aggregatum* ($2n = 2x = 38$).

The repetitive DNA probe DmoF14 identified dispersed fluorescent signals of different intensity on all chromosomes of the *Dendrobium* species (Fig. 5A, C, E). In *D. moschatum* and *D. aphyllum*, DmoF14 is widely scattered over most chromosome arms detectable as interstitial signals (Fig. 5A, C). Furthermore, 12–14 subtelomeric signals were observed in *D. aphyllum*. In *D. aggregatum*, almost half of the signals included pericentromeric regions (Fig. 5E).

The microsatellite probe DmoO11 consisting of GA dinucleotides was located on all chromosomes in *D. aphyllum* and *D. aggregatum* and on most chromosomes in *D. moschatum* (Fig. 5B, D, F). The majority of strong signals were found in interstitial regions of *D. moschatum* chromosomes (Fig. 5B). In *D. aphyllum* DmoO11 was dispersed over all chromosome arms with stronger hybridization in some interstitial sites (Fig. 5D). In *D. aggregatum* most of the hybridization of DmoO11 was confined to 2–3 interstitial sites per chromosome (Fig. 5F).

Six of eight clones of the $c_0t$*-1* plasmid library contained telomeric repeats. As a representative clone, DmoB22 has been used as a FISH probe. Metaphase chromosomes of *D. moschatum*, *D. aphyllum* and *D aggregatum* showed an identical hybridization pattern, revealing the presence of telomeric sequences at all chromosome termini (Fig. 5G–I). Of note, in *D. aphyllum* very strong hybridization signals were detected in an internal region of two chromosomes, while two strong signals were present at an intercalary position of another chromosome pair (Fig. 5H).

### *Physical mapping of ribosomal genes*

FISH was applied to localize the tandemly arranged 18S–5·8S–25S rRNA and 5S rRNA genes on *Dendrobium*

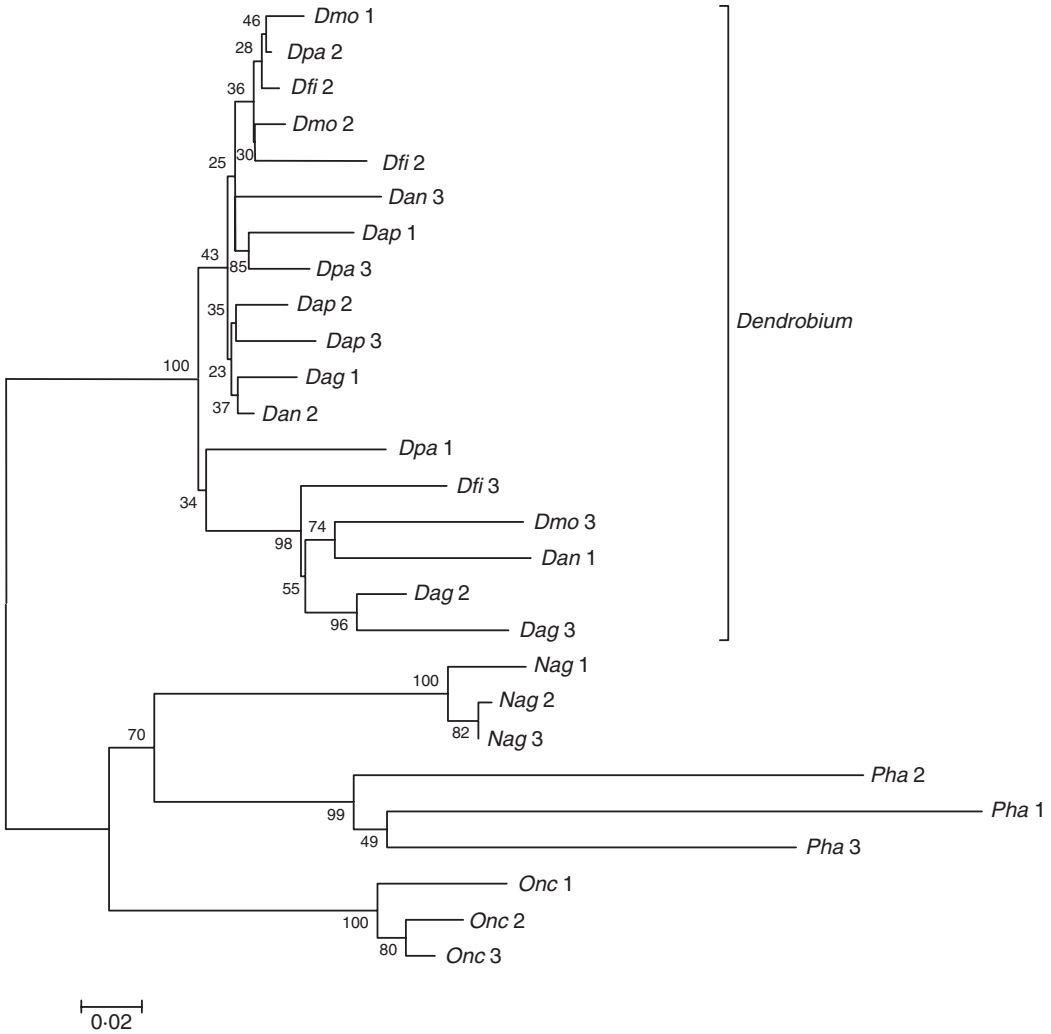


FIG. 4. Neighbour-joining analysis showing the sequence divergence of three DmoF14 repeats amplified from each of the six *Dendrobium* species and the species of other orchid genera. The number of bootstrap trials was 1000, and the resulting bootstrap values are indicated on the respective branches. The length of branches within the dendrogram represents the distance between the DmoF14 sequences, while the scale bar indicates the number of substitution events per site.

chromosomes, using heterologous probes. The clone pTa71 from *Triticum aestivum* (Gerlach and Bedbrook, 1979) was used to detect 18S–5·8S–25S rDNA, while the probe pXV1 from *Beta vulgaris* (Schmidt *et al.*, 1994) enabled the localization of 5S rRNA.

In *D. moschatum* the 18S–5·8S–25S rRNA genes are located on three pairs of chromosomes (Fig. 5J, green fluorescence). Two major sites showing bright signals labelled almost completely a chromosome pair. Nevertheless, closer inspection of the pTa71 hybridization signals on these two chromosomes revealed the absence of pTa71 in telomeric/subtelomeric regions (Fig. 5K, arrows). Two other strong signals were restricted to one arm of another chromosome pair, while the minor pair of sites was located at the subtelomeric region of two other chromosomes (Fig. 5J). In contrast, *D. aphyllum* showed only two sites of 18S–5·8S–25S rRNA gene signals (Fig. 5M, green fluorescence). The signals were localized in the terminal position of one chromosome pair and showed not fully condensed ribosomal gene arrays (Fig. 5M). In *D. aggregatum* a major 18S–5·8S–25S rRNA gene site was also observed in the terminal position of one chromosome pair (Fig. 5N, green signals). Additionally, minor sites were detected on the same pair of chromosomes (Fig. 5N, arrows).

FISH for the detection of 5S rRNA genes revealed two signals in *D. moschatum*, *D. aphyllum* and *D. aggregatum* (Fig. 5L–N; red fluorescence). In each orchid species investigated, the chromosomal position of the 5S rRNA gene was different. Two sites were located in an intercalary region in *D. moschatum* (Fig. 5L). In *D. aphyllum* and *D. aggregatum* the two 5S rRNA gene signals were observed in the terminal region of two other chromosomes (Fig. 5M, N).

## DISCUSSION

The genus *Dendrobium* comprises approx. 1200 species, but only a few of these species have been characterized regarding genome size (Jones *et al.*, 1998; Leitch *et al.*, 2009).

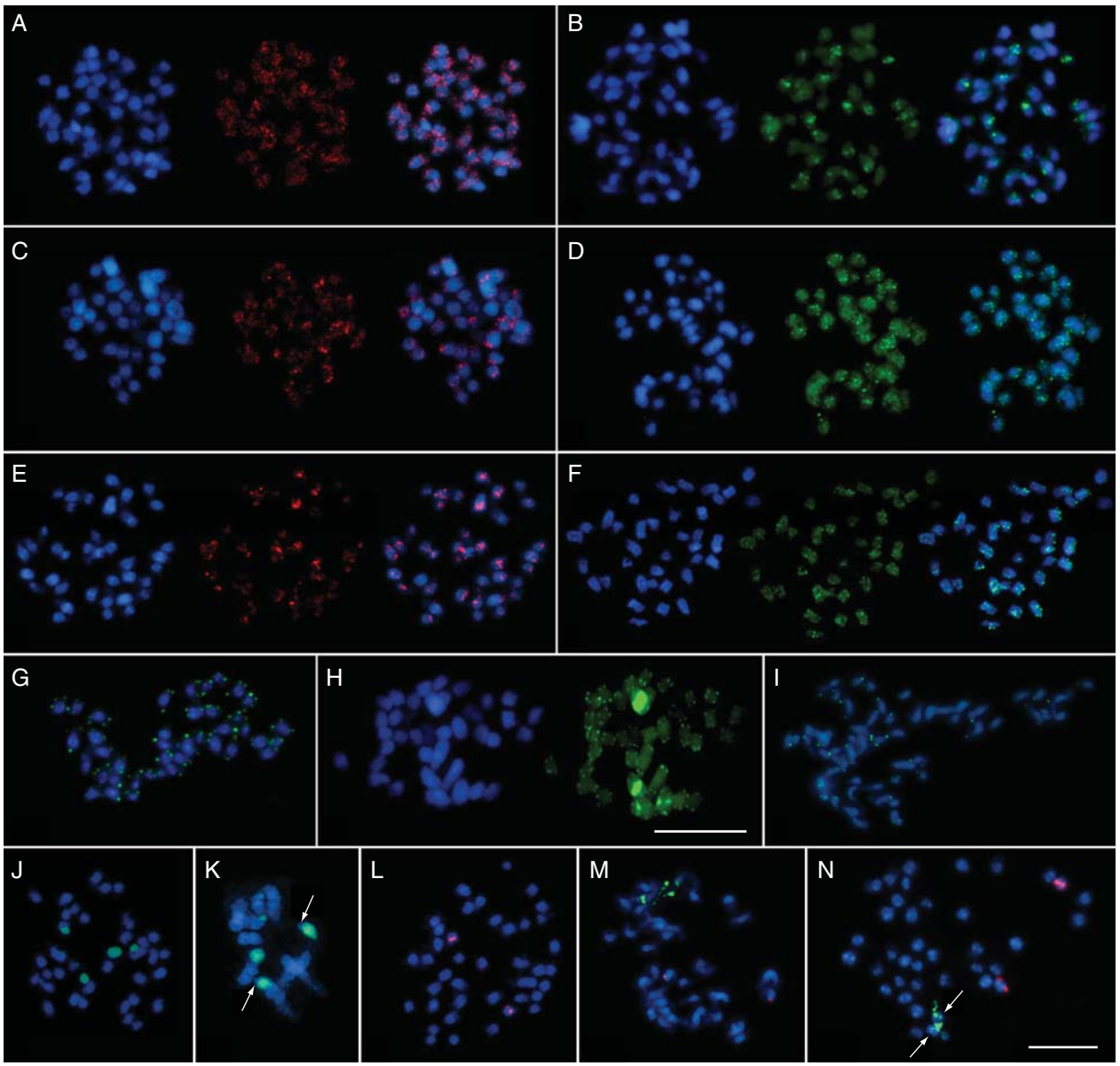


FIG. 5. Fluorescence *in situ* hybridization to metaphase chromosomes of three *Dendrobium* species. In panels A–F, the DAPI-stained DNA (blue fluorescence) shows the morphology of the chromosomes. The images on the left show the dispersed distribution of the DmoF14 fragment on the chromosomes of *D. moschatum* (A), *D. aphyllum* (C) and *D. aggregatum* (E), in red fluorescence (middle and overlay image in each panel). The images on the right show the dispersed localization of the microsatellite DmoO11 on the chromosomes of *D. moschatum* (B), *D. aphyllum* (D) and *D. aggregatum* (F), respectively, as green fluorescence (middle and overlay image in each panel). (G–I) Chromosomal localization of the telomeric sequence DmoB22 (green fluorescence) on chromosomes of *D. moschatum*, *D. aphyllum* and *D. aggregatum*, respectively. The internal positions of telomeric sequence motifs on two pairs of *D. aphyllum* chromosomes are visible as bright yellow–green signals (H, right). (J–N) rDNA FISH analysis on *D. moschatum* (J, K and L), *D. aphyllum* (M) and *D. aggregatum* (N) metaphases. Red signals indicate 5S rDNA repeats and green signals represent 18S–5·8S–25S rRNA arrays. Terminal regions lacking 18S–5·8S–25S rRNA genes on one pair of *D. moschatum* chromosomes are indicated by arrows in a partial metaphase (K, close-up). The minor 18S–5·8S–25S rRNA site on two *D. aggregatum* chromosomes is shown by an arrow in N. The scale bar in N corresponds to 10 μm and is valid for all images except H containing an individual 10 μm scale bar.

Molecular studies are available describing *Dendrobium*-specific rDNA sequences (Cheng *et al.*, 2004; Tsai *et al.*, 2004). However, thus far, there is no information regarding the abundance and chromosomal distribution of rDNA and other repetitive DNA families in *Dendrobium*. The present paper describes the isolation of repetitive sequences from *Dendrobium moschatum*, and comparative Southern analyses in other *Dendrobium* species and species from three different orchid genera.

Repetitive sequences can be isolated by several strategies. Restriction satellites are detectable as prominent bands after gel electrophoresis based on conserved recognition sites in repeating units. Other methods include ultracentrifugation with complexing dyes or shotgun cloning. Genome sequencing with next-generation technologies is becoming an increasing method for the detection of families of repetitive DNA, but these technologies have not yet been applied to *Dendrobium*

species. In order to identify *Dendrobium*-specific satellite repeats, 13 restriction enzymes with recognition sites covering four to six nucleotides with AT- or GC-rich motifs were tested. However, no prominent fragments of the typical size of satellite DNA (150–180 bp or multimers thereof) were detected following gel electrophoresis.

A rapid and efficient method for the identification of repetitive DNA is the isolation of $c_0t$-1 DNA, which is enriched for repeated sequences. This method has been used for the isolation of highly abundant repeats in many plant species (Yuan *et al.*, 2003; Hribová *et al.*, 2007; Anamthawat-Jonsson *et al.*, 2009). Based on a small *D. moschatum* $c_0t$-1 plasmid library, a telomere-like motif, a $(GA)_n$ microsatellite and a dispersed repeat with no homology to EMBL/GenBank entries were identified. The abundance of these repeats indicates the suitability of $c_0t$-1 DNA for further isolation of highly repetitive orchid DNA sequences.

Microsatellites are useful markers to assess the organization of genomes and their diversity on the species and population level in many higher plant species (Bell and Ecker, 1994; Becker and Heun, 1995; Röder *et al.*, 1995). It has been shown that different microsatellites are major components of the repeated DNA fraction of plant genomes (Schmidt *et al.*, 1993; Schmidt and Heslop-Harrison, 1996). In orchids, several studies aiming at the isolation and application of microsatellites have been undertaken in order to investigate intra- and interspecific variation (Pellegrino *et al.*, 2001; Helena *et al.*, 2008). Yue *et al.* (2006) developed simple sequence repeat markers for *Dendrobium* species, but their study included only hybrids, mostly selected as ornamental plants. Here microsatellites were isolated from *D. moschatum* that consisted of a perfect array of GA dinucleotides repeats. Although polymorphic hybridization patterns were not detected, it is noteworthy that the abundance of GA dinucleotides varies considerably between *D. moschatum*, *D. aggregatum* and *D. aphyllum.* This suggests that it might be possible to differentiate *Dendrobium* species and accessions by DNA fingerprinting, although the level of intra- or interspecific polymorphism has to be tested and more microsatellite motifs should be included. If Southern hybridization is used, the level of resolution depends on the combination of microsatellite motif and restriction enzyme, often resulting in contrasting hybridization patterns in different plant species (Schmidt and Heslop-Harrison, 1996; Schmidt *et al.*, 2000).

With FISH, a genome-wide $(GA)_n$ distribution has been observed, particularly in *D. aphyllum*. The position of microsatellites included interstitial and pericentromeric chromosomal regions. The relatively uniform coverage of the chromosomes is complemented by a local clustering in some regions as observed in *D. moschatum*. The distribution pattern is dispersed and similar to that reported in *Beta vulgaris* (Schmidt and Heslop-Harrison, 1996). In contrast, Hudakova *et al.* (2001) reported $(GA)_n$ sequences in centromeric regions of barley chromosomes, suggesting that the chromosomal position of microsatellites is variable.

The dispersed repeat DmoF14 has been isolated from the $c_0t$-*1* DNA fraction of *D. moschatum* herein. Comparative studies of plant repetitive DNA sequences support the investigation of phylogenetic relationships between plant species (Kamm *et al.*, 1995; Orgaard *et al.*, 1995; Frello and Heslop-Harrison, 2000). Southern hybridization showed that DmoF14 is conserved and has a similar genomic organization in *D. moschatum*, *D. aphyllum*, *D. aggregatum* and other *Dendrobium* species such as *D. anosmum*, *D. fimbriatum* and *D. palpebrae*. Moreover, sequencing of Dmo14 repeats of species from different genera revealed genus-specific diversification, and the presence of DmoF14 in distantly related orchid genera suggests that this repeat is an ancient component of orchid genomes which has probably been present in the last common ancestor of the species investigated. The evolutionary origin of DmoF14 remains elusive. In the six *Dendrobium* species investigated, Southern hybridization revealed a strong *Bam*HI fragment of approximately 350 bp which is consistent with conserved *Bam*HI restriction sites at nucleotide positions 36 and 378 in DmoF14. Retrotransposons are ancient and highly amplified DNA sequences of plant genomes, and it is widely accepted that the majority of dispersed sequences in plant genomes are derived from retrotransposons, in particular from LTR (long terminal repeat) retrotransposons (Flavell, 1986). Therefore, it is possible that DmoF14 is part of or is derived from an LTR of an ancient or yet unknown orchid retroelement.

Telomeres consist in most mono- and dicotyledonous angiosperms, gymnosperms and bryophytes of the highly conserved *Arabidopsis*-type sequence repeats $(TTTAGGG)_n$ (Richards and Ausubel, 1988; Fuchs *et al.*, 1995), while some genera of the *Asparagales* have a different type of telomeric sequence (Adams *et al.*, 2001; Rotkova *et al.*, 2004).

Here, an *Arabidopsis*-type telomeric sequence was observed on the chromosome termini of three *Dendrobium* species by FISH. Of note, the telomeric probe in *D. aphyllum* detected additional strong signals at pericentromeric and intercalary positions on two pairs of chromosomes (Fig. 5H). Several mechanisms can be postulated to explain this chromosomal rearrangement. In the phylogeny of *D. aphyllum*, Robertsonian fusions might have occurred, resulting in two pairs of submetacentric to metacentric chromosomes. The ancestral chromosomes could have been two acrocentrics (Schubert, 1992), but chromosomes with other position of the centromeres are also possible ancestors. However, as dicentric chromosomes are not stable, one centromere of the fused chromosomes must have been functionally inactivated. It is well known that Robertsonian fusions played an important role in altering karyotypes of animals and plants and therefore are a widespread mechanism in speciation (Meyne *et al.*, 1990; Cox *et al.*, 1993; Fuchs *et al.*, 1995). However, internal telomeric repeats indicating a Robertsonian fusion have not been detected in *D. aggregatum* (having $2n = 38$ chromosomes). Alternatively, the internal telomeric arrays can also result from DNA repair mechanisms, which has been observed in many plant species (Fitzgerald *et al.*, 2001). The telomerase binds to short homologous stretches in single strand gaps and synthesizes short telomeric arrays. It is known that di- to trinucleotide homologies are sufficient to act as telomerase substrate. Subsequently, newly synthesized telomeric repeats could have been amplified by slippage replication. Another scenario might involve either para- or pericentric inversion as a result of a breakage in the telomeric block at the end of the chromosomes, with a part of the broken telomere array being transferred to an internal position. Similarly, an

inversion of a chromosome arm or part thereof can transfer the telomeric block to an internal position requiring the healing of the newly formed chromosome end by telomerase activity, as described for wheat (Tsujimoto *et al.*, 1999). Nevertheless, more *Dendrobium* species need to be analysed for the presence of internal telomeric sequences to further unravel the mechanisms leading to ectopic positions of telomere repeats on *Dendrobium* chromosomes.

In addition to other repetitive sequence classes, the localization of rRNA gene arrays on plant chromosomes by FISH has been widely used (Leitch and Heslop-Harrison, 1992; Schmidt *et al.*, 1994), with both the 5S rRNA and the 18S–5·8S–25S rRNA genes providing useful markers for chromosome identification and karyotyping (Doudrick *et al.*, 1995; Brown *et al.*, 1999). In orchids just a few cytogenetic studies have been performed. Chromosome staining has been applied to show karyotype variation in *Phalaenopsis* and the related species *Doritis pulchirrima* (Kao *et al.*, 2001). Schwarzacher *et al.* (1980), Schwarzacher and Schweizer (1982) and Koehler *et al.* (2008) used banding techniques for karyotype analyses in Austrian *Cephalanthera* species and species of the genus *Christensonella*, respectively. The genomic organization and relationships in *Phalaenopsis* species were investigated by genomic *in situ* hybridization (Lin *et al.*, 2005). Telomeric sequences and rDNA genes were mapped on chromosomes of *Cephalanthera* species (Moscone *et al.*, 2007). The chromosomal positions of 5S rRNA and the 18S–5·8S–25S rRNA genes have also been analysed in some wild orchids and hybrids from Italy, and in species of the genus *Maxillaria* (Demerico *et al.*, 2001; Cabral *et al.*, 2006).

Here a comparative analysis of the distribution of rRNA gene arrays on chromosomes of three *Dendrobium* species was performed. In *D. moschatum*, *D. aphyllum* and *D. aggregatum*, the number of 18S–5·8S–25S and 5S rDNA sites was clearly determined. Apart from one chromosome pair in *D. moschatum*, all rDNA sites are at terminal positions. This particular *D. moschatum* chromosome pair showed a very strong FISH signal, suggesting that most of the DNA consists of 18S–5·8S–25S rRNA genes, which is probably the result of rRNA gene array expansion. In synthetic *Nicotiana* hybrids it has been shown that rDNA arrays undergo rapid changes including expansion or reduction (Kovarik *et al.*, 2008). However, each of the three *Dendrobium* species analysed here has a characteristic number and position of rDNA sites enabling the unequivocal identification of single chromosomes in a complement of the small and numerous orchid chromosomes.

Further analyses will need to prove the suitability of rDNA probes as well as probes from repetitive sequence families using the $c_0t$-1 DNA library of *D. moschatum* as a resource for genome analysis in the genus. Nevertheless, more *Dendrobium* species should be included in comparative molecular–cytogenetic analyses to provide a valuable complementation to taxonomic data based on morphological characters.

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