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Complex rearrangements are involved in *Cephalanthera* (Orchidaceae) chromosome evolution

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Abstract

The genus *Cephalanthera* is an excellent plant group for karyotype evolution studies because it exhibits a dysploid series and bimodal karyotypes. With the aim of understanding their chromosomal and phylogenetic relationships, rRNA genes and the *Arabidopsis*-type telomeric sequence were mapped by fluorescence *in-situ* hybridization (FISH), and the rDNA intergenic spacer (ITS) was sequenced for the first time in three European species: *C. longifolia* (2n = 4x = 32), *C. damasonium* (2n = 4x = 36) and *C. rubra* (2n = 4x = 44). One 45S and three 5S rDNA sites are observed in *C. longifolia*, one 45S and two 5S sites in *C. damasonium*, and two 45S and one 5S site in *C. rubra*. Telomeric signals were observed at every chromosome end in all three species and *C. damasonium* also displays interstitial signals on three chromosome pairs. In agreement with chromosome data, molecular analyses support *C. longifolia* and *C. damasonium* as closely related taxa, while *C. rubra* stands apart. Possible pathways of karyotype evolution are discussed in reference to a previous hypothesis. The results indicate that complex chromosomal rearrangements, possibly involving Robertsonian fusions and fissions, loss of telomeric repeats, gain or loss of rDNA sites and other heterochromatic sequences and inversions, may have contributed to generating the present-day karyotypes.

Electronic supplementary material
The online version of this article (doi:10.1007/s10577-007-1174-6) contains supplementary material, which is available to authorized users.

Introduction

Cephalanthera Rich. is a small genus of Orchidaceae, arguably the largest angiosperm family, and comprises about 14 terrestrial species from temperate regions of the northern hemisphere. It is included in the tribe Neottieae, a monophyletic and comparatively primitive group of orchids whose flowers are characterized by the presence of a single fertile anther and soft pollinia (cf. Tutin *et al*. 1980, Walters *et al*. 1984, Dressler 1993, Dressler & Chase 1995). Cytogenetic data reported for 12 *Cephalanthera* species suggest a paleopolyploid origin and show a wide variation in chromosome number within the genus as follows (highly derived polyploid numbers in parentheses and basic numbers in square brackets): 2n = 32 (48, 64) [x = 8], 34 (68) [17], 36 (54) [9], 42 [7], and 44 [11], with x = 8 being the most common basic number (cf. plant chromosome number indexes, main references in Fedorov (1969), Moore (1973), Goldblatt (1985)). Furthermore, as typical for *Neottieae*, the genus exhibits extreme karyotype asymmetry, with bimodal complements consisting of two size classes of chromosomes – two to four chromosome pairs are quite large and the remaining pairs are comparatively small (Kliphuis 1963, Meili-Frei 1966, Tanaka & Yokota 1982, Mehra & Kashyap 1983, Lee & Kim 1986, D'Emerico *et al*. 1999, 2000). *Cephalanthera* is, therefore, an attractive plant group for investigating karyotype evolution.

The two white-flowered, paleotetraploid species *C. longifolia* (2n = 32) and *C. damasonium* (2n = 36) have similar bimodal karyotypes consisting of three large chromosome pairs and a gradient of smaller chromosomes, while the red-flowered *C. rubra* (2n = 44) has a less extreme bimodal karyotype. Detailed studies using Feulgen staining, Giemsa C-banding and DNA-binding fluorochromes show significant differences in both heterochromatin composition and distribution between the two white-flowered species (Schwarzacher & Schweizer 1982, Schwarzacher 1983). These authors suggested a model involving Robertsonian-type fission or fusion events leading to the karyotype differentiation between *C. longifolia* and *C. damasonium*.

In order to test, refine and extend the proposed hypothesis, we now employ molecular cytogenetic and DNA sequence analyses in three species growing in Europe: *C. longifolia* (L.) Fritsch, *C. damasonium* (Mill.) Druce and *C. rubra* (L.) Rich. FISH enables the physical mapping of sequences to their location within the genome, in particular repetitive sequences that cannot be mapped easily by any other method (see Schwarzacher 2003). Repetitive sequences change rapidly during evolution, providing excellent markers for the identification of chromosomes and chromosome segments, and for following evolutionary chromosome rearrangements. The 5S and 45S rRNA genes, in particular, have been used extensively to establish possible chromosomal homeologies (e.g., Moscone *et al*. 1999, Adams *et al*. 2000, Liu *et al*. 2003, Taketa *et al*. 2005, Cai *et al*. 2006, Hasterok *et al*. 2006). Another universal probe is the largely conserved telomeric sequence that lends itself as an ideal marker for the identification of chromosome ends and, therefore, aids in revealing chromosomal rearrangements related to changes in chromosome number (Meyne *et al*. 1990, Lee *et al*. 1993, Fuchs *et al*. 1995). While the coding regions within the rRNA genes are conserved among species, the ITSs show rapid changes in DNA sequence composition. Thus, they provide a good measurement of evolutionary time and species relation in taxonomic and ecological studies, as has also been shown in Orchidaceae (Cox *et al*. 1997, Bateman *et al*. 2003). In this paper, we have thus complemented the cytogenetic data by analysing the systematic relationship between the three species by molecular phylogenetic assessment of ITS sequences.

Materials and methods

Plant material

C. longifolia (L.) Fritsch, *C. damasonium* (Mill.) Druce and *C. rubra* (L.) Rich. were collected in Lower Austria in May and June 2000. For details of the materials' provenance, see Schwarzacher & Schweizer (1982). Herbarium specimens were deposited in the Herbarium WU of the Institute of Botany, University of Vienna. *Epipactis palustris* (L.) Crantz and *E. helleborine* (L.) Crantz, used as outgroups for phylogenetic analysis, were obtained from the Botanical Garden of the University of Vienna in 2001.

Chromosome preparation

Young flower buds, longitudinally sliced, were pre-treated with 0.05% colchicine for 11–21 h at 4°C, or with 0.002 M 8-hydroxyquinoline for 19–21 h at 4°C or for 2–4 h at 4°C followed by 7–17 h at 16°C. The buds were then fixed in freshly prepared methanol–glacial acetic acid (3:1) and stored at −20°C until preparation. Somatic chromosome spreads from ovules were prepared according to Schwarzacher *et al*. (1980), except that, before squashing in a drop of 45% acetic acid, ovaries were macerated in an enzyme solution (2% w/v cellulase Onozuka R-10 (from *Trichoderma viridae*; Serva Electrophoresis, Heidelberg, Germany) plus 20% v/v pectinase (from *Aspergillus niger*; Sigma-Aldrich, Vienna, Austria) in 0.01 M citric acid–sodium citrate buffer, pH 4.8) for 25 min at 37°C.

DNA probes for FISH

45S rDNA, 5S rDNA and telomeric repeats were localized using the following DNA probes: R2, a 6.5 kb fragment of an 18S-5.8S-25S (45S) rDNA repeat unit from *Arabidopsis thaliana*, including internal transcribed spacers ITS1 and ITS2 and a short 5′ segment of the intergenic region (IGR) (Wanzenböck *et al*. 1997); pXV1, a 349 bp fragment of the 5S rRNA gene repeated unit from *Beta vulgaris* (Schmidt *et al*. 1994); D2, a 5S rDNA clone from *Lotus japonicus* (Pedrosa *et al*. 2002); and an *Arabidopsis*-like telomeric probe, amplified by PCR according to Ijdo *et al*. (1991), using the oligomer primers $(5'\text{-TTTAGGG-}3')_5$ and $(5'\text{-CCCTAAA-}3')_5$. Clone R2 and the telomeric probe were labelled with Cy3-dUTP (Amersham, Vienna, Austria), and pXV1 and D2 were labelled with biotin-14-dATP (Roche, Vienna, Austria), all by nick translation using the kit of Roche Diagnostics. As expected, both 5S rRNA probes gave comparable results (the same signal strength and position) on test preparations.

Fluorescence in-situ hybridization (FISH)

Pre-treatment of the preparations was performed following Pedrosa *et al*. (2001). Chromosome and probe denaturation, *in-situ* hybridization, post-hybridization washes and detection were carried out after Heslop-Harrison *et al*. (1991), with the modifications described in Pedrosa *et al*. (2002). Hybridization mixes consisted of 50% (v/v) formamide, 10% (w/v) dextran sulfate, 2× SSC (saline sodium citrate: 0.3 M NaCl plus 0.03 M sodium citrate) and 2–5 ng/μl of probe. The biotin-labelled probes were detected using ExtrAvidin-fluorescein isothiocyanate (FITC) conjugate (Sigma) in 1% (w/v) bovine serum albumin. Preparations were counterstained and mounted with 2 μg/ml DAPI in Vectashield (Vector, Burlingame, CA, USA). Reprobing of slides for localization of different DNA sequences on the same cell was performed according to Heslop-Harrison *et al*. (1992). Photographs were taken on a Zeiss Axioplan fluorescence microscope equipped with a mono cool view CCD camera (Photometrics, Tucson, AZ, USA). Black and white images taken with the different filter combinations were combined and pseudo-coloured using the IPLab Spectrum software (IPLab, Fairfax, VA, USA). Digital images were imported into Adobe Photoshop version 9.0 for final processing using only those functions that are applied to all pixels of the image.

Karyotype analysis

Four to five metaphase plates after FISH and DAPI staining of each species were used for chromosome measurements. The arm ratio [$r = q$ (long arm length)/p (short arm length)] was used to classify the chromosomes as recognized by Levan *et al*. (1964) with the modifications introduced by Schlarbaum & Tsuchiya (1984): *m*, metacentric ($r = 1.00$–1.34); *msm*, meta-submetacentric ($r = 1.35$–1.69); *sm*, submetacentric ($r = 1.70$–2.99); *st*, subtelocentric ($r = 3.00$–7.00); and *t*, telocentric ($r > 7.00$). Measurements of arms (and bands, where applicable) from homologous chromosomes were used to calculate average values of relative length. In the case of intercalary loci/bands, their position was calculated as a percentage of arm length from the centromere ($di = d \times 100/a$, where d = distance of band centre from the centromere, a = length of the corresponding chromosome arm) (Greilhuber & Speta 1976). In the idiograms, the chromosomes were arranged according to decreasing length except in a few cases where indistinguishable chromosomes were grouped. The three to four largest chromosome

pairs of each species were labelled with the same numbers as in Schwarzacher & Schweizer (1982). Telomeric signals and non-constant heterochromatic bands that did not aid chromosome identification were not included in the idiograms, although the latter were considered in the calculations of heterochromatin amount.

PCR amplification and sequencing of rDNA ITS region

Total genomic DNA was extracted from material stored at −80°C, as well as from herbarium specimens (*C. rubra*), following the 2X CTAB procedure of Doyle & Doyle (1987). The ITS region, containing the internal transcribed spacers ITS1 and ITS2, and the 5.8S gene, comprising 780–794 bp, was amplified by PCR using available universal primers (Baldwin *et al*. 1995). The highest yields of PCR products were achieved using a 100 μl PCR reaction containing 2.5 units of Taq DNA polymerase (Promega, Mannheim, Germany), 2 mM magnesium chloride, 0.2 mM of each dNTP, 0.25 μM of each primer, and 2–8 ng of template total DNA in the reaction buffer provided by the supplier. Amplified fragments were checked on a 1% agarose gel and the fragments were purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany).

The purified fragments were directly sequenced on an ABI 377 automated sequencer (PE Applied Biosystems, Vienna, Austria) using dye terminator chemistry following the manufacturer's protocols. Two cycle sequence reactions were performed for each template using each of the two primers for PCR amplification. The software 'Sequence Navigator' and 'Auto Assembler' (PE Applied Biosystems) were used to edit and assemble the complementary sequences.

Sequence alignment and phylogenetic analyses

Alignments were obtained using the software Clustal V (Higgins *et al*. 1992) and adjusted visually. Phylogenetic analysis was performed using PAUP beta test version 4.ob10 (Swofford 2002). A heuristic search was conducted with 1000 random sequence additions and tree bisection-reconnection (TBR) branch swapping, permitting 10 trees to be held at each step. Confidence limits for the tree were assessed by performing 1000 replicates of bootstrapping (Felsenstein 1985), with simple sequence addition and TBR swapping.

Results

Comparative karyotyping

Karyotype formula and length, mean chromosome length, nuclear DNA content, heterochromatin amount and distribution, and number and position of 45S and 5S rDNA loci are summarized for each species of *Cephalanthera* in Table 1. Illustrations of somatic metaphases after FISH and DAPI staining appear in Figure 1, and idiograms of the respective species are presented in Figure 2 (data from metaphase complement measurements are available as supplementary data).

In general, karyotype data derived from DAPI-stained chromosomes after FISH of the three species are in agreement with previous contributions from this laboratory, where detailed chromosome measurements after Feulgen staining and discussion with results from other authors are given (Schwarzacher & Schweizer 1982, Schwarzacher 1983). However, FISH allowed the identification of additional chromosomes with similar morphology; thus, the grouping of homologous chromosome pairs for calculating mean lengths and arm ratios was facilitated. *Cephalanthera longifolia* (2n = 4x = 32) and *C. damasonium* (2n = 4x = 36) have three chromosome pairs (nos. 1–3) that are 2- to 4-fold larger (10.5–14.5 μm) than the remaining chromosomes of the complement (pair nos. 4–16 and 4–18, respectively), which are comparatively small and of decreasing size (2.5–6.0 μm). These extreme bimodal karyotypes are reflected in the large standard deviation of the mean chromosome lengths in Table 1. In *C. rubra* (2n = 4x = 44), karyotype bimodality is less evident as it has two very large (nos. 1–2, 10.5–12.0 μm in size), two comparatively large (nos. 3–4, 8.0 μm) and 18 medium- to small-sized chromosome pairs of decreasing length (nos. 5–22, 2.5–6.0 μm). Submetacentric chromosomes are predominant in the three species, and, in general, the largest and the smallest chromosomes of the complement have a comparatively high arm ratio (Figures 1 and 2). In all species, total karyotype length is rather similar and correlates with nuclear DNA content in the case of *C. longifolia* and *C. damasonium*, where data are available (Bennett & Leitch 1995). In addition, chromosome number correlates positively with karyotype length and negatively with mean chromosome length (Table 1).

Table 1. Karyotype features of *Cephalanthera* species

Species[a]	2n	Haploid karyotype formula (n)	Mean haploid karyotype length (μm) (SD)	Mean chromosome length (μm) (SD)	DNA amount 1C (pg)[b]	DAPI-positive heterochromatin amount (%) and distribution[c]	No. and position of rDNA sites[d]	
							45S	5S
C. longifolia (5)	32	$3m + 2msm + 6sm + 4st + 1t$	94.54 (9.53)	5.91 (3.36)	16.8	5.99 (*ce*, *pce*)	1 (3q)	3 (6p, q; 11q)
C. damasonium (4)	36	$5m + 2msm + 6sm + 3st + 2t$	104.73 (8.76)	5.82 (3.36)	16.4	6.00 (*ce*, *pce*)	1 (2p)	2 (4p; 16q)
C. rubra (5)	44	$4m + 5msm + 8sm + 3st + 2t$	112.95 (2.44)	5.13 (2.59)	—	8.46 (*ce*, *pce*, *te*)	2 (1q; 3q)	1 (3q*)

Abbreviations: SD, standard deviation; *p* and *q*, upper (short) and lower (long) arm, respectively, in the idiograms (Figure 2); *m*, metacentric; *msm*, meta-submetacentric; *sm*, submetacentric; *st*, subtelocentric; and *t*, telocentric chromosome; *ce*, centromeric; *pce*, paracentromeric; and *te*, telomeric band.

[a]Figures in parentheses indicate the number of somatic metaphases analysed per species.

[b]Values taken from Bennett & Leitch (1995). The genotypes used in DNA measurements are not necessarily identical to those in this study.

[c]Heterochromatin amount expressed as a percentage of the haploid (n) karyotype length.

[d]The corresponding chromosome arms involved are indicated in parentheses. Synteny of 5S site with 45S site is denoted by an asterisk.

Heterochromatin was visualized by DAPI as positive bands after FISH and reached similar amounts in all species (although slightly higher in *C. rubra*), with values 38% lower than those previously reported by Giemsa C-banding in the case of *C. longifolia* and *C. damasonium* (Schwarzacher & Schweizer 1982). There is a common heterochromatic banding pattern characterized by the presence of centromeric and paracentromeric bands, and the main heterochromatic regions are the (para)centromeric bands of pairs nos. 1, 2 and 4 of *C. longifolia*, pairs nos. 1 to 3 of *C. damasonium* and pairs nos. 2, 4 and 8 of *C. rubra* (Figure 2). Notably, the heterochromatic band on chromosome pair no. 1 of *C. damasonium* comprises the whole short arm (Figures 1c and 2). Bands in pair no. 4 of *C. longifolia* and no. 3 of *C. damasonium* are not visible in all metaphase spreads, as well as some terminal bands in *C. rubra* and the centromeric heterochromatin of most chromosomes. The heterochromatic banding pattern between homologous chromosomes is highly homomorphic in the three species analysed, except in pair no. 4 of *C. longifolia* where the large centromeric band, which extends on the long arm, occurs most commonly in the heterozygous condition (Figures 1 and 2; see also Schwarzacher & Schweizer 1982).

Cytological mapping of the rRNA genes and the telomeric sequences by FISH

The FISH patterns of the two rRNA gene families, 5S and 45S, vary between species. One pair of 45S rDNA loci is present in *C. longifolia* and *C. damasonium*, and two pairs are present in *C. rubra*, while one pair of 5S rDNA loci is found in *C. rubra*, two pairs in *C. damasonium* and three pairs in *C. longifolia*. In *C. rubra*, the 5S site pair is syntenic with one of the 45S site pairs. All 45S loci are located at a telomeric position, while 5S sites are located at either intercalary or terminal positions (Figure 1).

In *C. longifolia*, one chromosome of the haploid complement (no. 3) bears a 45S site at the telomeric region of the long arm, while two other chromosomes (nos. 6 and 11) bear 5S loci. Two 5S sites are terminally located on both arms of chromosome no. 6, the one on the long arm being smaller. A third 5S cluster is interstitially located on the long arm of chromosome no. 11 (Table 1, Figures 1a and 2). In

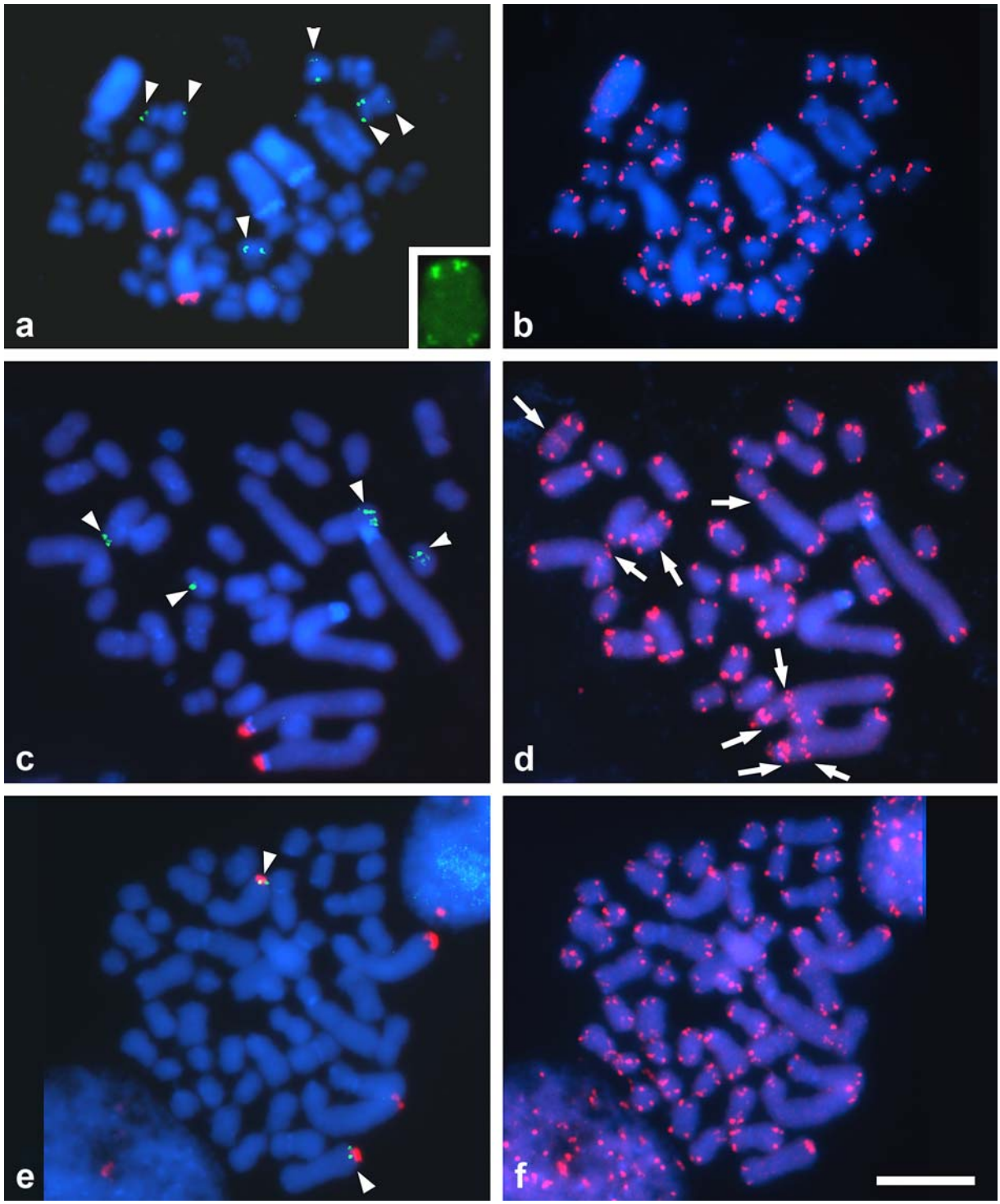

Figure 1. FISH to metaphase chromosomes of three *Cephalanthera* species using probes for the 45S and 5S rRNA genes, as well as for telomeric sequences. (a, b) *C. longifolia* (2n = 32). (c, d) *C. damasonium* (2n = 36). (e, f) *C. rubra* (2n = 44). Hybridization sites of the 45S (red signals) and 5S probes (green signals) are shown in (a, c and e). Hybridization sites of the telomeric probe (red signals) in the same cells as in (a, c and e) are shown in (b, d and f), respectively (one chromosome in (d) was lost). Chromosomes were counterstained with DAPI (blue). Arrowheads indicate 5S rDNA sites. Arrows indicate ectopic telomeric signals. Inset in (a) shows an example of a chromosome no. 6 of *C. longifolia* with 5S rDNA sites on both arms from another cell. Bar represents 10 μm.

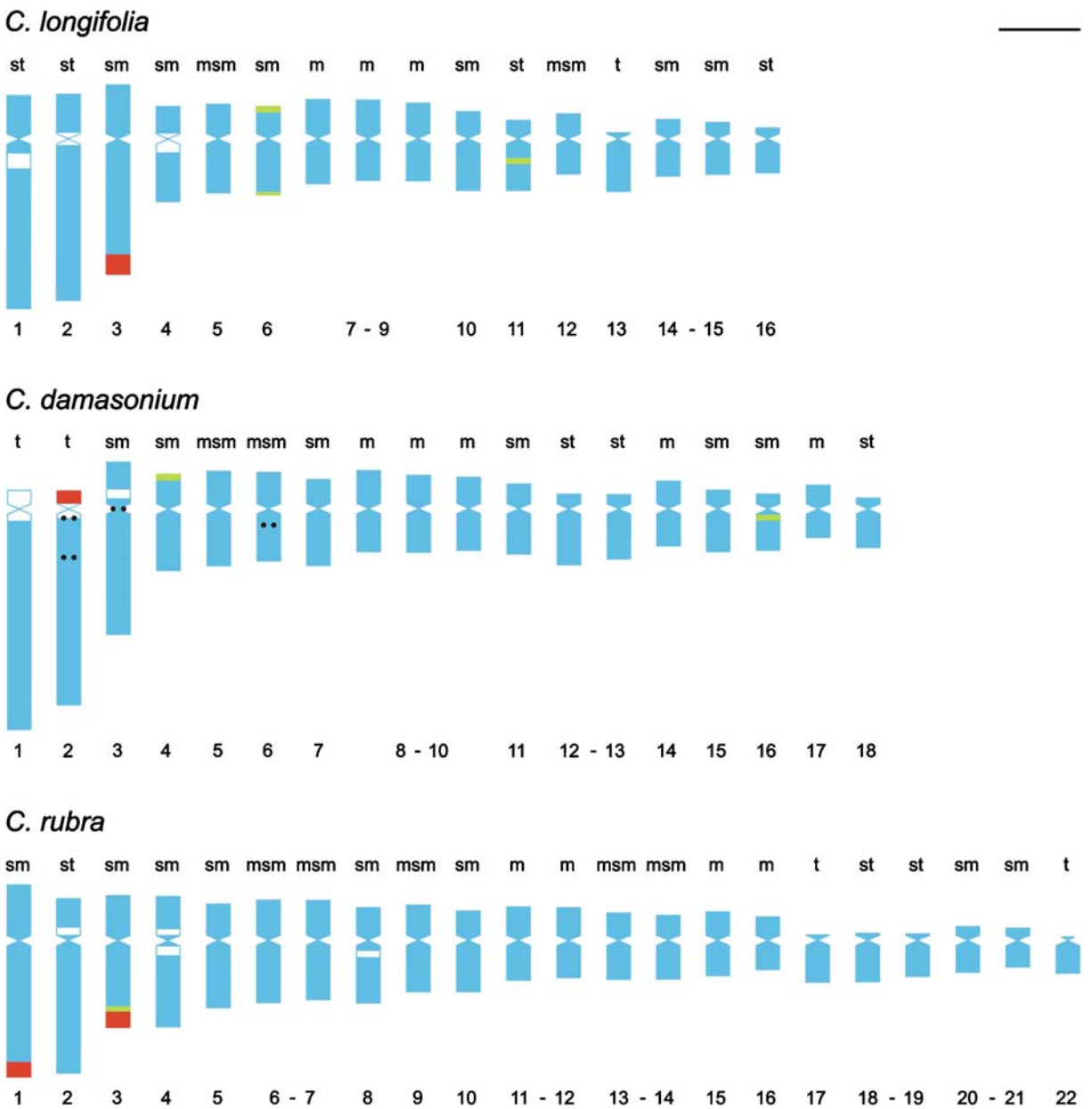

Figure 2. Idiograms of three *Cephalanthera* species showing chromosome lengths and types, DAPI fluorescence banding pattern (DAPI-positive heterochromatic bands are in white and euchromatic regions are shown in blue), and the physical mapping of three repetitive sequences using FISH—5S (green) and 45S rRNA loci (red), and interstitial telomeric sequences (black). The chromosomes are arranged, in general, according to decreasing length. In the different species, chromosomes that have the same number on the idiograms are not necessarily homologous. Chromosomes of similar morphology and size that lack characteristic landmarks are arranged in groups. Bar represents 5 μm.

C. damasonium, one chromosome pair (no. 2) possesses a 45S locus distally located, which comprises nearly the entire short arm. Another two pairs, nos. 4 and 16, display 5S loci of rather similar length, the first one at a telomeric position of the short arm and the second in the paracentromeric region of the long arm (Table 1, Figures 1c and 2). In *C. rubra*, two chromosome pairs (nos. 1 and 3) carry a 45S rDNA cluster of similar size at a telomeric position on the long arm. In addition, one of these pairs (no. 3) has

an intercalary 5S cluster on the long arm, adjacent to the 45S site (Table 1, Figures 1e and 2).

All taxa exhibit the *Arabidopsis*-like telomeric sequence at every chromosome end (Figure 1b,d,f). Additionally, *C. damasonium* has telomeric repeats at centromeric and intercalary positions, i.e., two loci—one paracentromeric and another intercalary—on the long arm of chromosome no. 2, one locus at the centromere of chromosome no. 3 and one interstitial locus on the long arm of chromosome no. 6 (Table 1, Figures 1d and 2).

Sequence analysis of the rDNA ITS region

Among the members of tribe Neottieae, *Epipactis* is one of the closest genera to *Cephalanthera* according to cladistic analysis based on morphological features and plastid sequences; both taxa also display natural intergeneric hybrids (Dressler 1993, Kores *et al.* 1997, Freudenstein & Rasmussen 1999, Cameron *et al.* 1999, Cameron 2004). Thus, *E. palustris* and *E. helleborine*, both with 2n = 40, were chosen as the outgroup for the phylogenetic analysis carried out in this study.

The ITS region, including the internal transcribed spacers ITS1 and ITS2, and the 5.8S gene, comprised 780–794 bp, with ITS1 and ITS2 being approximately 248 bp and 245 bp long, respectively. The sequences obtained for the analysed region in each species were deposited in GenBank under the following accession numbers: *C. longifolia*, AY146447; *C. damasonium*, AY146446; *C. rubra*, AY146445; *E. palustris*, AY146448; and *E. helleborine*, EF153104. Of the total characters, 37 variable characters are parsimony uninformative and 65 are parsimony informative, where ITS1 was more variable than ITS2. A heuristic search generated a single most parsimonious tree with 115 steps

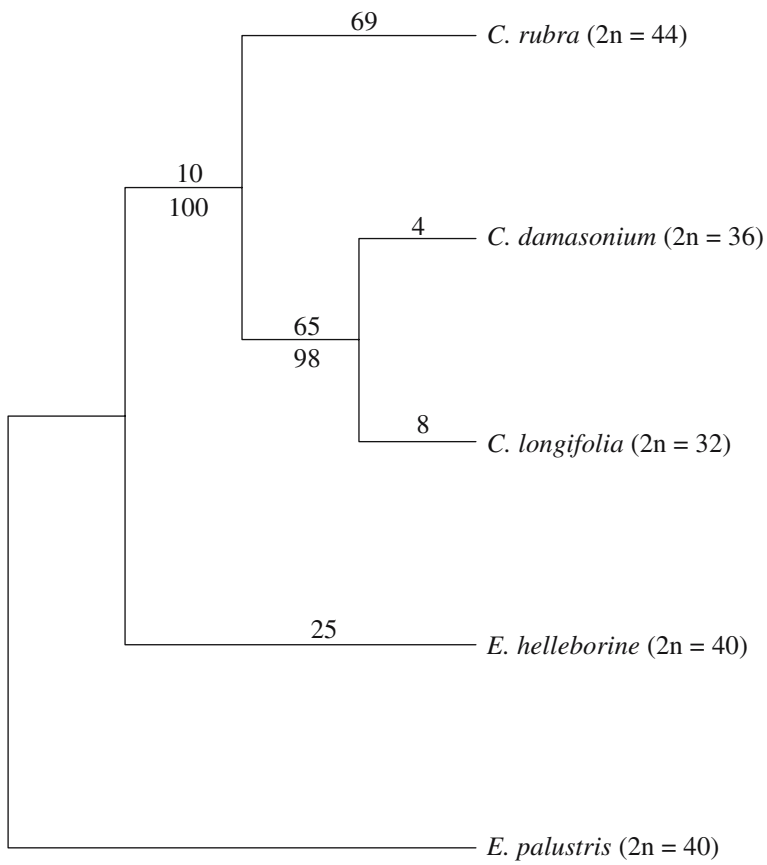

Figure 3. Single most parsimonious tree obtained with ITS sequences of three *Cephalanthera* species and two outgroup *Epipactis* species. Values above and below are branch lengths and bootstrap percentages (>50%), respectively.

having a consistency index (CI) of 0.96 and a retention index (RI) of 0.93. The phylogenetic tree with the calculated Fitch lengths (ACCTRAN optimization) and bootstrap values is given in Figure 3. The species *C. longifolia* and *C. damasonium* form a clade with a strong bootstrap value (98%). *C. rubra* is quite distinct and well separated from the other two species. There were roughly 12 mutations between *C. longifolia* and *C. damasonium*, whereas 142 mutations were observed between *C. longifolia* and *C. rubra*, including a few base pair deletions in *C. rubra*.

Discussion

The present study is focused on testing our proposed model of karyotype evolution in *Cephalanthera* (Schwarzacher & Schweizer 1982), with particular reference to the species pair *C. longifolia* and *C. damasonium*, by cytogenetic mapping of the rRNA coding genes and the telomeric sequences, which is performed for the first time in the genus. The results obtained demonstrate that the FISH mapping of the referenced sequences, in combination with the phylogenetic analysis of the ribosomal ITS sequences, is a powerful approach for interpreting the relationships between *Cephalanthera* species and for contributing arguments to evolutionary considerations.

Phylogenetic relationships inferred from ITS sequencing data

Although comprehensive molecular phylogenetic analyses using internal transcribed spacers of the nuclear rRNA genes as well as plastid sequences have been performed in several genera of orchids (Bateman *et al*. 2003, Cameron *et al*. 1999, Cameron 2004), *Cephalanthera* as a whole has still not been considered in molecular phylogenetic analyses. The rapidly evolving nuclear ITS region has been successfully used to investigate species-level questions in Orchidaceae (Bateman *et al*. 2003). The most parsimonious tree obtained from our data on the nuclear rDNA ITS sequences demonstrates that *C. longifolia* and *C. damasonium* are closely related species, while *C. rubra* is more distant, in agreement with the obtained cytogenetic data.

Cytogenetic characterization and karyotype relationship between species

The cytogenetic methodology applied in the present work allowed us to gain valuable karyological data for species characterization. The use of DAPI after FISH differentially stained the heterochromatin in all three *Cephalanthera* species (see Schwarzacher & Schweizer 1982), as has been observed in other plant groups (Moscone *et al*. 1996, 1999). The precise position of the rDNA sites in the three *Cephalanthera* species, which could not be determined with silver staining (Schwarzacher & Schweizer 1982), is now established.

FISH patterns of ribosomal RNA genes vary between the *Cephalanthera* species, mostly in reference to the number and chromosomal distribution of the signals. The number of 5S and 45S rRNA gene clusters reported in plants by FISH is highly variable, with many species exhibiting more 5S sites in comparison to 45S sites, as in *C. damasonium*, while many others display more 45S loci, as in *C. rubra* (see Moscone *et al*. 1999, Adams *et al*. 2000, Taketa *et al*. 2005, Cai *et al*. 2006, Hasterok *et al*. 2006). It should be noted that the appearance of 5S loci in both arms of a chromosome pair, as we found in *C. longifolia*, was rarely observed in other plants (Shibata & Hizume 2002, Taketa *et al*. 2005, Hasterok *et al*. 2006), and is probably due to an inversion-type rearrangement with breakpoints at subtelomeric regions, one of them within the 5S cluster (see Figure 4b). In *Vicia sativa*, two 5S rDNA sites were on opposite chromosome arms as in *C. longifolia*, but in this species both loci were observed in pericentromeric, not terminal, locations (Navratilova *et al*. 2003). The presence of 45S rDNA sites in terminal regions of both chromosome arms seems to be more common (Zhang & Sang 1998, Pedrosa *et al*. 2003, Vaio *et al*. 2005), possibly due to its more frequent presence at terminal chromosome sites.

After rRNA gene mapping and DAPI banding, some possible chromosome homeologies in the studied *Cephalanthera* species could be established: i.e. (1) chromosomes no. 6 of *C. longifolia* and no. 4 of *C. damasonium*, both submetacentric of similar size and with a 5S site on the short arm at a telomeric position, the former also with another 5S locus in the telomeric region of the long arm; (2) chromosomes no. 11 (subtelocentric) of *C. longifolia* and no. 16

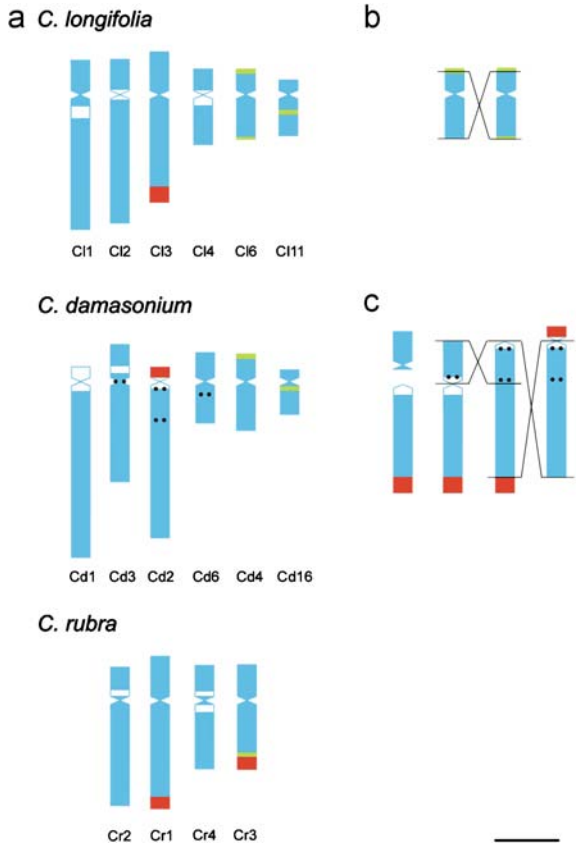

Figure 4. Possible chromosome homologies and rearrangements in *Cephalanthera*. (a) Chromosomes of *C. longifolia*, *C. damasonium* and *C. rubra* arranged according to possible homologies. Only chromosomes easily identifiable by chromosome markers are depicted. (b,c) Hypothetical origin of chromosome no. 6 of *C. longifolia* (b) and of chromosome no. 2 of *C. damasonium* (c). Bar represents 5 μm.

(submetacentric) of *C. damasonium*, both with a 5S locus intercalarily located on the long arm—the difference in chromosome size may be due to pericentromeric heterochromatin amplification; and (3) chromosomes no. 3 of *C. longifolia* and no. 1 of *C. rubra*, both submetacentric of equal length and with a 45S site on the long arm at a telomeric position (Figure 4a). In general, karyotypic features demonstrate that *C. longifolia* and *C. damasonium*, although differentiated by chromosome number, appear as closely related taxa with similar mean chromosome length and heterochromatin amount, a large chromosome pair bearing a 45S site and two small pairs with 5S loci. On the other hand, *C. rubra* appears more distant in having a less extreme bimodal karyotype with a higher chromosome number and heterochromatin amount, terminal heterochromatic bands in the short arm of most large to medium-sized chromosome pairs, and two comparatively large chromosome pairs with 45S sites, one of them also carrying a 5S locus. These results are in agreement with the obtained ITS sequencing data.

Karyotype evolution

Schwarzacher & Schweizer (1982) demonstrated by fluorescent staining and Giemsa C-banding that chromosome and heterochromatin repatterning has accompanied the evolution of the *Cephalanthera* species considered here, which are supposed to be paleotetraploids with $x = 8$ (*C. longifolia*), 9 (*C. damasonium*) and 11 (*C. rubra*). Although the development of the *C. rubra* karyotype could not be hypothesized, two alternative paths for the karyotype differentiation of *C. damasonium* and *C. longifolia* had been proposed (Schwarzacher & Schweizer 1982, their Figure 11). One possibility is that $x = 8$ represents the ancestral stage, where a polyploidization event gave rise to a karyotype consisting of four large and 12 small chromosomes. Subsequently, a partial loss of material from one of the large chromosomes caused the creation of a set of three large and 13 small chromosomes. From this, one branch could have lead to the $2n = 32$ karyotype of *C. longifolia* by heterochromatin amplification, while another branch could have given rise to the $2n = 36$ karyotype of *C. damasonium*, by a Robertsonian fission of two of the large chromosomes. A second possibility is that the ancestral basic number was $x = 9$, from which the *C. damasonium* karyotype was derived, while the karyotype of *C. longifolia* could have been developed later by Robertsonian fusions.

Robertsonian changes are probably the most important source of karyotype repatterning in animals but their occurrence in higher plants seems to be less frequent (Jones 1998). Through the application of classical approaches, chromosome rearrangements of this kind have been inferred in the evolution of Orchidaceae genera belonging to the subfamily Cypripedioideae (Cox *et al*. 1997, 1998) and the tribe Neottieae (i.e., *Epipactis*, *Listera* and *Cephalanthera*; Schwarzacher & Schweizer 1982, D'Emerico *et al*. 1999, 2000). Nevertheless, more conclusive evidence has been lacking. Our findings of four ectopic localizations of the telomeric sequence in the karyotype of *C. damasonium*, including centromeric

signals in two large chromosomes, is noteworthy. The presence of telomeric signals in centromeres of particular, large chromosomes is often thought to be the remnant of chromosomal rearrangements, particularly Robertsonian fusion events that occurred during genome evolution in animals and plants (Meyne *et al*. 1990, Cox *et al*. 1993, Fuchs *et al*. 1995).

The presence of centromeric loci of telomeric repeats on two large chromosomes in *C. damasonium* suggests that Robertsonian fusions may have played a role in the evolution of the genus. The particular situation of chromosome pair no. 2 of this species, with two interstitial telomeric loci in the long arm, could be explained by the occurrence of at least two rearrangements. A Robertsonian fusion could have given rise to the centromeric site with a consequent change in chromosome number; additionally, a pericentric inversion involving the short-arm telomere could have been the origin of the more distally placed locus in the long arm, with a consequent change in chromosome morphology (see Figure 4c).

However, although *C. longifolia* has the lowest chromosome number among the investigated species, no intercalary telomeric sites were observed in this species. Therefore, two main possibilities have to be considered. First, that Robertsonian fusions were also responsible for the reduction in chromosome number in *C. longifolia*, but, in this species, the centromerically located telomeric sequences have been lost. In this sense, Cox *et al*. (1993) also failed to identify interstitial telomeric repeat sequences that may have remained from fusion events in Orchidaceae and other plant families. Telomeric sequences in ectopic localizations may become free of function and could possibly decrease in number through mechanisms commonly involved in microsatellite evolution (Azzalin *et al*. 2001). Furthermore, if Robertsonian fusions do not include telomeres they cannot be detected by FISH using the telomeric probe. Any of these possibilities could account for the absence of interstitial telomeric signals in *C. longifolia*, despite their presence in *C. damasonium*. Second, the interstitial telomeric repeats detected in *C. damasonium* chromosomes are not a result of centric fusions, but have been incorporated into centromeric and intercalary heterochromatin by other means. Interstitial telomeric sequences could arise, for example, from double-stranded DNA break repair mediated by telomerase or integration through mobile elements (Azzalin *et al*. 2001) and have been detected interspersed within non-telomeric repetitive DNA in interstitial and centromeric sites in *Arabidopsis thaliana*, *Lotus japonicus*, *Pinus densiflora*, tomato, potato and other plant species (Richards *et al*. 1991, Presting *et al*. 1996, Pedrosa *et al*. 2002, Tek & Jiang 2004, Shibata *et al*. 2005). If this is the case in *Cephalanthera*, other structural rearrangements have been responsible for the reduction or increase in chromosome number in this genus.

Independently of the origin of the centromerically located telomeric loci and the nature of the structural rearrangements responsible for the chromosome number change, our present results indicate that complex chromosomal rearrangements are involved in the evolution of this group. In order to explain the differences in chromosome morphology and repetitive sequence distribution in the three species, additional rearrangements need to be postulated, such as gain or loss of rDNA sites and other heterochromatic sequences and inversions (see Figure 4). Further karyotype analyses by FISH with telomeric sequences, rRNA genes and other chromosomal markers as probes should be carried out in other *Cephalanthera* species and related genera to gain a more comprehensive view of the chromosome evolution of this group.

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