Proceedings
The 4th International
Carnivorous Plant Conference
Tokyo, Japan 2002 ©

A-2

A molecular cytogenetic study in *Drosera filiformis* and *D. graminifolia*

Kazuko Futagawa, Rie Suzuki and Katsuhiko Kondo

Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, 1-4-3 Kagamiyama, Higashi-Hiroshima City 739-8526, Japan

Abstract. Relationships between *Drosera filiformis* distributed in the South-eastern United States and *D. graminifolia* distributed in the Eastern Brazil were studied and compared by molecular cytogenetics using fluorescence *in situ* hybridization (FISH) with the probes of 45S ribosomal DNA (rDNA; by pTa71) and the *Arabidopsis*-type telomere sequence repeats. The two species had quite different orcein-stained karyotypes as well as chromosome numbers and exhibited 45S rDNA signals commonly at the terminal region of chromosomes but different signal numbers that showed no correlation. FISH using the *Arabidopsis*-type telomere sequence repeats indicated multi-minute signals occurred lineally on the chromosome along the gap between the sister chromatids as well as the both telomere regions of chromosome. Additionally, the chromosomes of *D. filiformis* showed another signals of the *Arabidopsis*-type telomere sequence repeats on the interstitial region and other regions *at random*. *Drosera filiformis* and *D. graminifolia* could be originated from different source and direction within the genus and are not closely related to each other.

Introduction

Among the members of the Droseraceae, *Drosera* is the largest genus and consists of somewhat 90～140 species (*e.g.*, Diels, 1906; Schlauer, 1996). A half of the species of the genus, ca 65 species have shown their chromosome numbers and karyotypes (*e.g.*, Hoshi and Kondo, 1998a, b). Kondo and his group (*e.g.*, Kondo *et al.*, 1976; Kondo and Lavarack, 1984; and so on in Kondo's early references) have discovered that *Drosera* chromosomes have diffused-centromeric habits. This was very first finding and only a case in the dicot plants. The series of our chromosomes researches in *Drosera* have been approving this peculiar phenomenon; light microscopic observation always showed no constrictionon the chromosomes observed (a lot of papers); γ -ray exposure produced a few to numerous fragments of chromosomes which were all survived since they supposedly had own centromeres (Sheikh *et al.*, 1995; Furuta and Kondo, 2000); Centromere banding technique always showed diffused-centromeric habits (Sheikh *et al.*, 1995), scanning electron microscopic observation showed masses of microtubules or spindle fibers and no primary constriction (Nontachaiyapoom *et al.*, 2000). Beside *Drosera* chromosomes, Aldrovanda chromosomes were speculated to have diffused centromeric chromosomes however, it was rather difficult to detect details in diffused-centromeric habit in *Aldrovand* chromosomes because of their too small chromosomes (Hoshi and Kondo, 1998a).

Chromosome sizes in *Drosera* are correlated with distribution locations of species (Hoshi and Kondo, 1998a, b). Australian *Drosera* shows well chromosomal diversity as well as morphological diversity but the *Drosera* species endemic to South Africa always show simply and commonly 2n=40 small chromosomes (Kondo and Oliver, 1979; Hoshi and Kondo, 1998; Nicholas and Kondo, 1998). However, chromosome observation in South American species of *Drosera* is very much lacking in most standard references.

Gene mapping on chromosome by FISH promises chromosome identification, analysis of chromosome structures, species relationships, and so on.

Materials and Methods

Drosera filiformis, D. graminifolia (Fig. 1) and their close relatives; total seven species used in this study are tabulated in Table 1. The reasons why *D. filiformis* and *D. graminifolia* were chosen for the main materials in this study are that (1) both species have quite similar leaf morphology of filiform, (2) different, allopatric distributions, and (3) different sizes of chromosomes.

They were all axenic-cultured in vitro on 1/2 strength of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1.5% sucrose, 0.3% Gelrite and no growth substance at pH 5.5 under environment at 23℃ under 1200 lux light intensity for 24 hours illumination.

The root tips harvested were pretreated in 0.002M 8-hydroxyquinoline at 18℃ for 2.5 hours and then, were fixed in ethanol-acetic acid (3:1) and stored at -4℃. They were treated with an ethanol series (70%, 35% and then, distilled water) for ten minutes each. Then, they were enzymatic-macerated in 3% cellulase Onozuka R10 (Yakult), 1.12% pectolyase Y-23 (Seishin) in the solution of 0.5 M EDTA and 2XSSC (1XSSC: 0.15 M NaCl+0.015M sodium citrate) at pH 4.5 at 37℃ for ca 30 minutes. After rinsed in distilled water for ten minutes, they were sealed with 45% acetic acid. The slides were placed in a freezer at -80℃ for five minutes or more and their cover-slips were removed for air-drying.

For orcein-staining, naked slides with preparations were added a few drops of 3% acetic-acid and were placed in a chamber moistened with 45% acetic acid for staining for five minutes.

The 18S-5.8S-26S rDNA of wheat, pTa71, which contained the spacer region, was used as 45S rDNA probe (Gerlach and Bedbrook, 1979).

The *Arabidopsis*-type telomere sequence repeats was produced by the polymerase chain reaction (PCR) method. The PCR reaction mixture consisted of 0.2 M dNTP (TAKARA), 1.25 units Taq polymerase (TAKARA Taq; TAKARA), 0.5 mM each of the primers of 5'-TTTAGGG-3' and 5'-CCCTAAA-3' and 1XPCR buffer (TAKARA). Cycles of the PCR were described as follows: The first cycle was denaturation at 95℃ for 90 seconds, annealing at 55℃ for 30 seconds and primer extension 75℃ for 30 seconds. After DNA amplified oligonucleotides were confirmed by electrophoresis, they were precipitated by ethanol and resuspended in TE solution.

The 45S rDNA and the Arabidopsis-type telomere sequence repeats were labeled by nick translation (Bionick labeling System: Life Technologies, GIBCO BRL).

The FISH method used was of Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University. The preparations were treated by 50 μl Rnase solution containing 0.1 mg/ml Rnase A (Sigma) in 2XSSC at 37℃ for one hour. After washing in 2XSSC for 15 minutes, they were refixed in 4% paraformaldehide dissolved in PBS at room temperature for ten minutes. The preparations were then washed in sterilized water and treated with an thanol series (70, 80, 100% five minutes each). After they were air-dried, 10 μl of the predenatured 45S rDNA or the *Arabidopsis*-type telomere sequence repeats probe mixture were applied to the slides and covered with silicone-coated cover-slips and sealed. The slides were denatured at 80℃ for three minutes and incubated at 37℃ overnight in Omnislide (HYBAID). They were washed in 2XSSC twice for ten minutes each. Then, they were dropped 70 μl of 1.5% bovine serum albumin (Sigma), 1.5% blocking reagent (Boehringer Mannheim) in 0.1 M Tris-HCl and 0.15 M NaCl at pH 7.0 and covered and sealed with a sealing film (Parafilm) for 15 minutes. The hybridization probes were detected by 20 μg/ml avidin-FITC conjugate (Sigma) and counterstained by 1 μg/ml propidium iodide (PI; Sigma). The slides were observed under fluorescence microscope using a blue filter (B-2A; Nikon). Microphotographs were taken on Super G400 (FUJI) film.

Since visualization of the signal of the *Arabidopsis*-type telomere sequence repeats had difficulty, signal amplification method was applied

Results and Discussion

Orcein-karyotyping The chromosome number of 2n=40 for *Drosera graminifolia* and that of 2n=20 for *D. sessilifolia* were recorded here for the first time. The chromosome number of 2n=20 found here was new to the South American species of *Drosera*. The chromosome numbers and sizes for the seven species of *Drosera* are tabulated in Table 3.

Centromeric regions in their chromosomes were not seen. Interphase chromosomes in all the species studied were commonly identified as "complex chromocenter type." The species in northern hemisphere always had single modality of chromosome sizes in their karyotypes at mitotic metaphase while *D. graminifolia* as well as *D. sessilifolia* had commonly bi-modality in their karyotypes consisted of two large chromosomes and the other similar almost the same sized chromosomes.

FISH using 45S rDNA The five species of *Drosera* studied commonly had 45S rDNA signals at the terminal region of chromosome (Figs. 2 and 3). The signal numbers found in the five species are tabulated in Table 4. This phenomenon supports Furuta and Kondo (1999); they observed the same phenomenon in *D. falconerii*. *Drosera graminifolia* (2n=40) and *D. sessilifolia* (2n=20) did not show any simple correlation with 45S rDNA signal numbers. *Drosera* of sect. Drosera commonly showed the 45S rDNA signals on certain middle-sized chromosomes, while *D. sessilifolia* of sect. Thelocalyx had the signals on larger-sized chromosomes (first, second, fifth and sixth chromosomes).

FISH using the *Arabidopsis*-type telomere sequence repeats Since this kind of signal in *Drosera* studied were so small and faint, signals were physically amplified by using secondary antibody method. Chromosomes of *D. filiformis* showed double dot signals of the *Arabidopsis*-type telomere sequence repeats near the terminal region, not exactly at the terminal region, and the interstitial region and furthermore chromosome region around the gap between the sister chromatids (Fig. 4). Chromosomes of *D. graminifolia* showed double dot signals at the both terminal regions of all of the chromosomes and single dot signals at the central region on the gap between the sister chromatids of 12 chromosomes (Fig. 4). According to Kondo and Furuta (1999) recorded signals of the *Arabidopsis*-type telomere sequence repeats in *D. falconerii* were dispersed throughout the chromosomes. On the other hand, diffused-centromeric chromosomes of monocot *Luzula luzuloides* showed the *Arabidopsis*-type telomere sequence repeats at the terminal region and sometimes at the interstitial region in order (Fuchs *et al.*, 1995). However, the present study showed chromosomes of *D. filiformis* and *D. graminifolia* had signals of the *Arabidopsis*-type telomere sequence repeats more or less in order. In case of *Allium cepa*, the terminal heterochromatic region of chromosomes could be covered by rDNA sequences or 375bp satellite DNA sequences to preserve chromosome terminal regions (Pich and Schubert, 1998). Drosera chromosomes with diffused centromeres may have some specific structures like *Allium cepa*. The signals in the chromosome of the Drosera species studied along the gap between the sister chromatids were seemed to be sometimes situated or conjugated in connection between sister chromatids.

As a conclusion, *D. filiformis* and *D. graminifolia* seemed to be originated from different strain and face to different evolutionary directions.

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Table 1. Materials used in this study

Species*	Distribution**	Source	Chromosome number (2n)	*in vitro* or *in vivo*
Subgen. Drosera				
Sect. Drosera L.				
Drosera brevifolia Pursh	Atlantic North America Brazil, Uruguay	Harsteal, U.S.A.	20	*in vitro*
D. capillaris Poir.	Atlantic North America Cuba,Guiana, Honduras		20	*in vitro*
D. filiformis Raf. var. *filiformis*	Atlantic North America	Nagoya, Japan cultiv.	20	*in vitro* and *in vivo*
D. graminifolia St. Hil.	Brazil, Guiana Venezuela	Caracas, Brazil	40	*in vitro* and *in vivo*
D. montana St. Hil.	Brazil, Venezuela	Itambe Peak, Brazil	40	*in vitro*
D. villosa St. Hil.	Brazil	Nagoya, Japan cultiv.	40	*in vitro*
Sect. Thelocalyx Planch.				
D. sessilifolia St. Hil.	Brazil, Guiana	U.S.A. cultiv.	20	*in vitro*

After *Seine and Barthlott (1994) and **Diels (1906)

Table 2. Differences between *Drosera filiformis* var. *filiformis* and *D. graminifolia*

Species	Leaves		Stipules	Seeds	Flowers	Habitat
	similarities	differences				
D. filiformis var. *filiformis*	threadlike leaves, red head glands on leaves,	up to 50cm long, glandular hairs with green stalks	fulvous lanuginose form, adnate basal leaves	ovoid and/or ellipsoid form, papillate testa	1.5cm across rose pink petal	peaty and sandy soil, savannah and margin of bogs
D. graminifolia	petioles indistinct, almost absent	over 30cm long, glandular hairs with red stalks	fuscuous membranous form, short acute dentate apex	narrowly fusiform	2cm across pink-lilac, red petal	rock and/or sand hills with wet surface

After Diels (1906), Schnell (1976) and Rivadavia (1996)

Table 3. Chromosome numbers and lengths of seven species of *Drosera* studied

Species	Chromosome number (2n)	Range of chromosome length (μm)
Drosera brevifolia	20	1.59-1.02
D. capillaris	20	2.48-1.55
D. filiformis var. *filiformis*	20	2.95-1.50
D. graminifolia	40	1.56-0.43
D. montana	40	0.93-0.45
D. villosa	40	1.09-0.56
D. sessilifolia	20	1.13-0.54

Table 4. Signal numbers in chromosomes of five species of *Drosera* studied by FISH using 45S rDNA probe

Species	Signal numbers		Chromosome number (2n)
	Interphase	Metaphase chromosomes	
Drosera brevifolia	1-7	2	20
D. capillaris	1-5	2	20
D. filiformis var. *filiformis*	1-6	4	20
D. graminifolia	1-8	6	40
D. sessilifolia	1-4	4	20

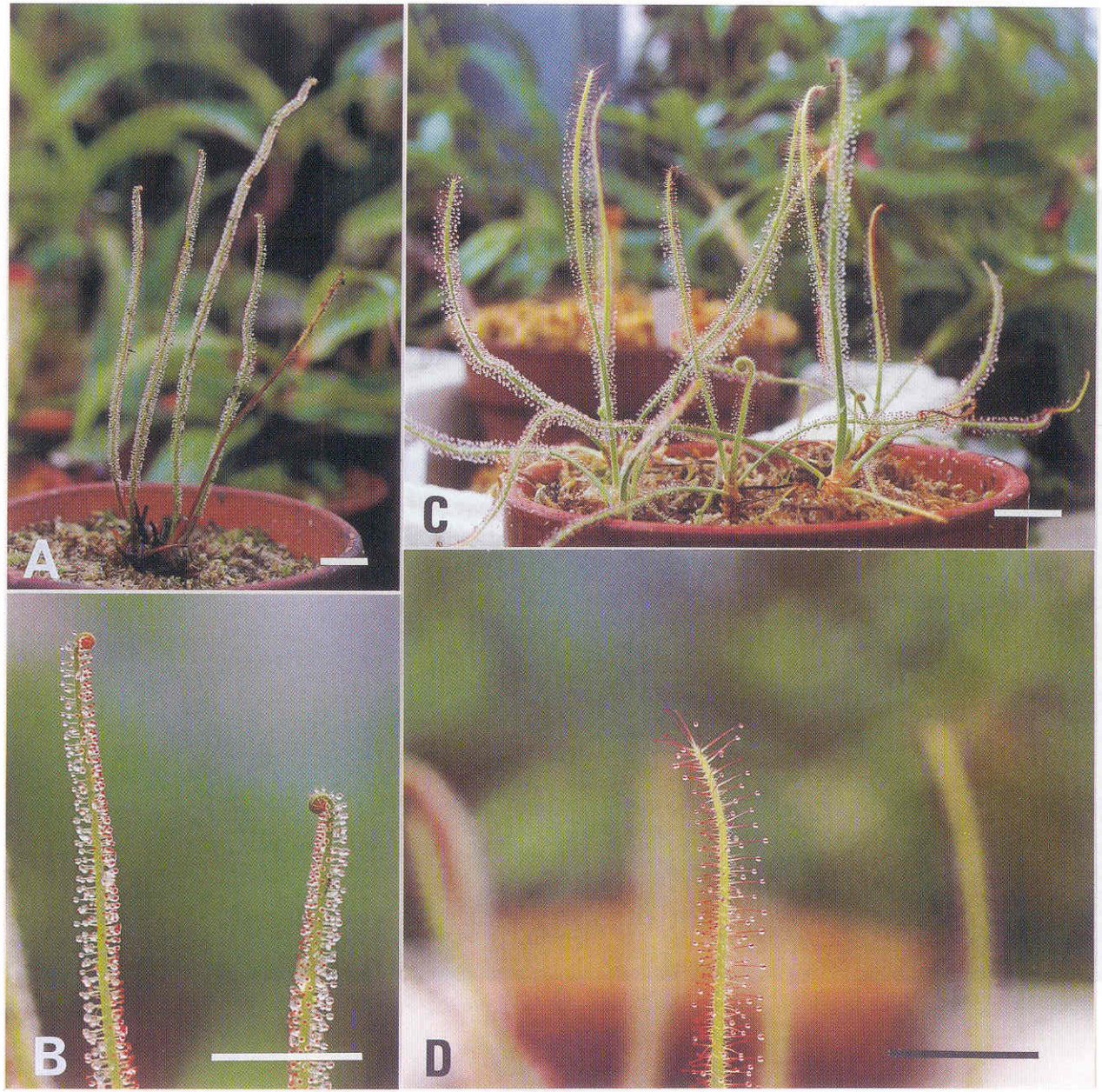

Figure 1. *Drosera filiformis* Raf. var. *filiformis* (A and B) and *D. graminifolia* St. Hil. (C and D)

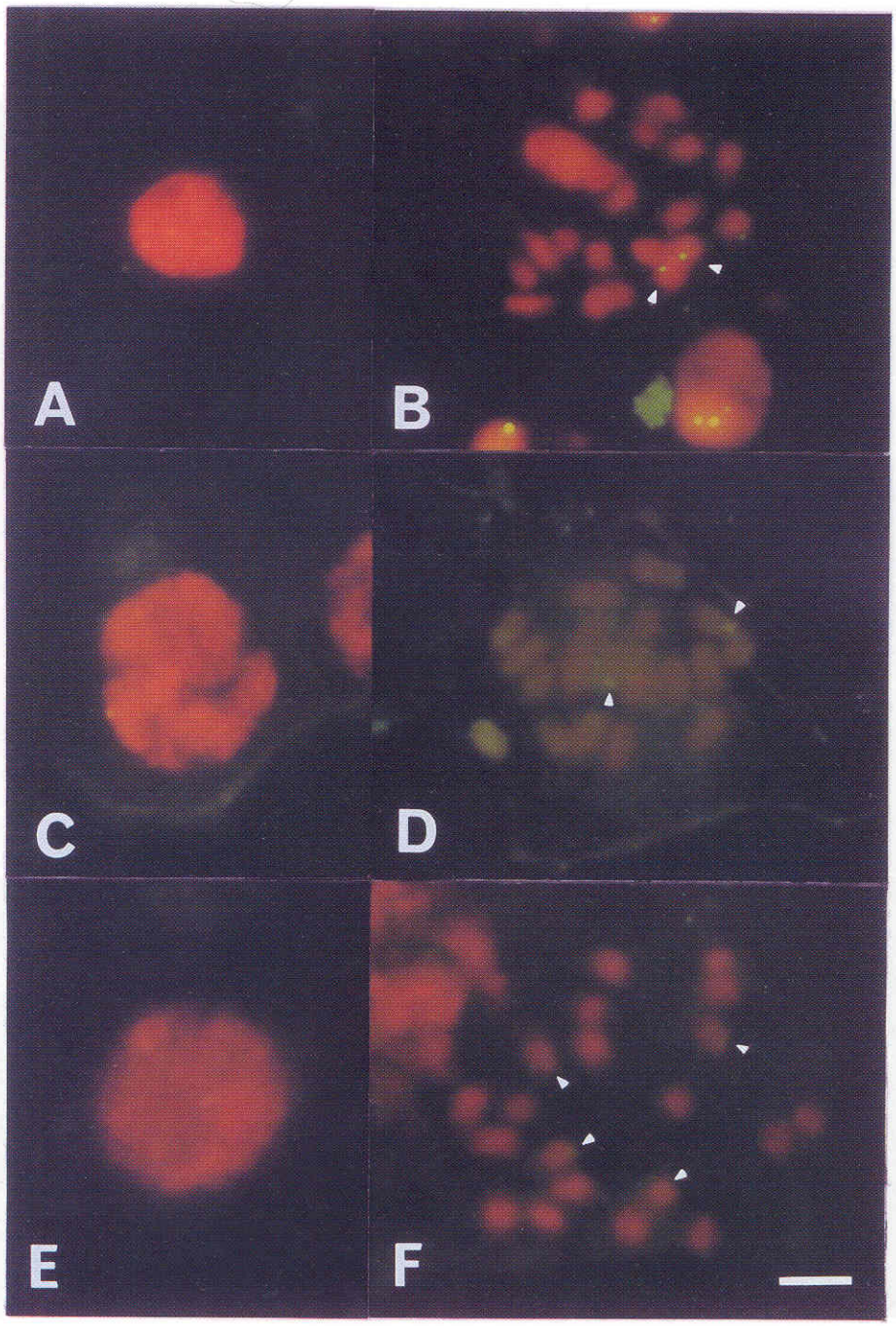

Figure 2. 45S rDNA signals by FISH in chromosomes of three species of *Drosera* in North America. A and B. *D. brevifolia*. C and D. *D. capillaris*. E and F. *D. filiformis* var *filiformis*. A, C, and E. Interphase chromosomes. B, D and F. Mitotic metaphase chromosomes. Bar=5 μ m.

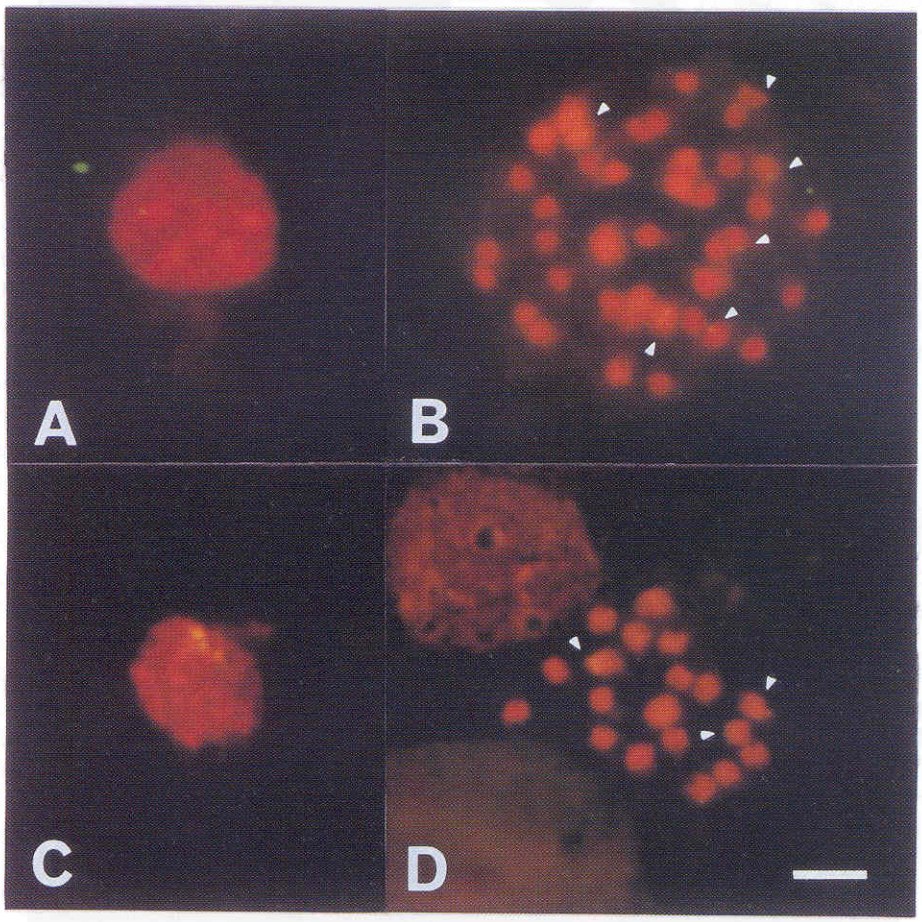

Figure 3. 45S rDNA signals by FISH in chromosomes of *Drosera graminifolia* (A and B) and *D. sessilifolia* (C and D) in South America. A and C. Interphase chromosomes. B and D. Mitotic metaphase chromosomes. Bar=5 μ m.

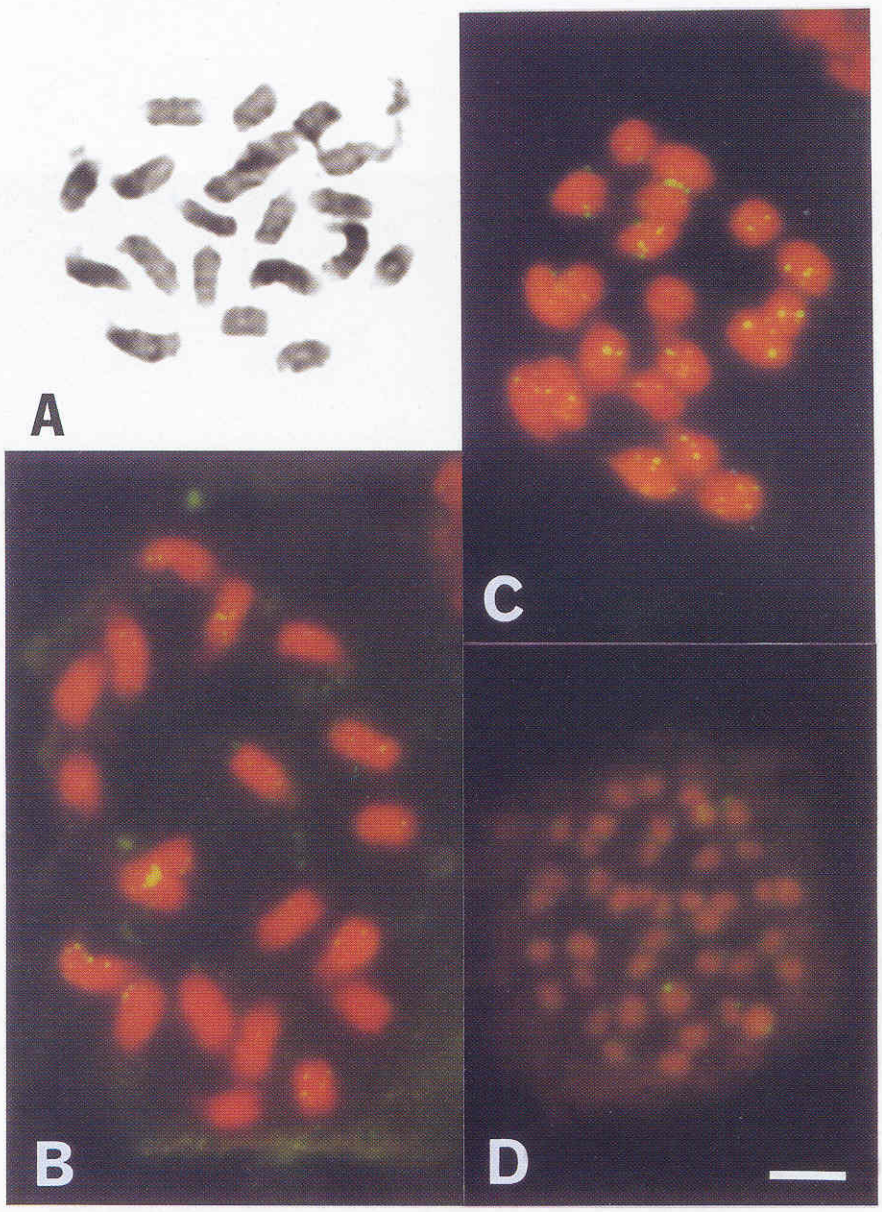

Figure 4. Diffused-centromeric natures of *Drosera* chromosomes analyzed by FISH using the *Arabidopsis*-type telomere sequence repeats. A. Orcein-stained chromosomes for comparison. B-D. Numerous small signals of the *Arabidopsis*-type telomere sequence repeats in mitotic mid-metaphase chromosomes. B and C. *D. filiformis* var. *filiformis*. D. *D. graminifolia*. Bar=5 μ m.