Chromosomal traits of *Chamaelirium luteum* (Melanthiaceae) with particular focus on the large heterochromatic centromeres

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ABSTRACT: Chromosomal morphology and behavior during the mitotic cell cycle and properties as to the DNA base composition in heterochromatin of *Chamaelirium luteum* from eastern North America were investigated by conventional and fluorescent banding methods. The chromosomes proved to have no primary constrictions; instead, they had unusually large heterochromatic monocentromeres termed "macrocentromeres" that were as thick as the chromosome arms and tended to protrude poleward at metaphase. It also became evident that the centromeres are rich in AT base pairs, whereas the satellites are GC-rich. Data suggested that the centromeric domains were tightly compacted almost through the cell cycle and situated at the nuclear periphery during telophase, interphase and prophase. The roles of centromeres and their causality to the property of centromeric DNA were discussed briefly. Some chromosomal traits of *C. luteum* were compared with those known for Asian congeners reported to have holocentromeres.

KEY WORDS: Centromeric DNA, *Chionographis*, Fluorescent chromosome banding, Holocentromere, Karyotype, Macrocentromere.

INTRODUCTION

Chamaelirium Willd. had long been treated as a monotypic genus represented by *C. luteum* (L.) A. Gray endemic to eastern North America. It was, however, recently combined by Tanaka (2017) with *Chionographis* Maxim. indigenous to east and southeast Asia, and the enlarged *Chamaelirium* (s.l.) currently comprises ten species. He divided the genus into two sections, *Chamaelirium* and *Chionographis* (Maxim.) N. Tanaka, and subdivided the latter into two subsections, *Cathayana* N. Tanaka and *Chionographis*. The populations of Asian species are reportedly hermaphroditic and/or gynodioecious (Tanaka, 1985, 2003, 2016, 2017; Maki, 1993), whereas those of *C. luteum* are dioecious (Gray, 1837; Meagher, 1980; Utech 2002). East Asian species, *C. japonicum* (Willd.) N. Tanaka, *C. koidzumianum* (Ohwi) N. Tanaka and *C. hisauchianum* (Okuyama) N. Tanaka (subsect. *Chionographis*), have been chromosomally investigated by several researchers (Sato, 1942; Hara and Kurosawa, 1962; Ajima, 1976; Tanaka and Tanaka, 1977a, 1979, 1980; Tanaka, 2020a, b). Some traits of their chromosomes, such as chromosome numbers, karyotypes at metaphase, and chromatin condensation/decondensation pattern during the mitotic cell cycle, have been reported. Among others, it is noteworthy that their chromosomes are reported to be holocentric (Tanaka and Tanaka, 1977a, 1979; Tanaka, 2020a, b). On the other hand, *C. luteum* has so far been less chromosomally studied. To date, its chromosome number $2n = 24$ (Kawano, 1976; Tanaka, 1985; Tanaka and Tanaka, 1985; Pellicer *et al*., 2014), which is shared by *C. japonicum* and *C. koidzumianum*, and the nuclear DNA amount (Pellicer *et al*., 2014) have been reported.

Tanaka (1985) and Tanaka and Tanaka (1985) preliminarily reported that *C. luteum* has monocentric chromosomes, but the details of it have been unpublicized until now. The present study was undertaken to fill the lack of knowledge on chromosomal traits of *C. luteum*, aiming to provide a basis for elucidating chromosomal relationships between *C. luteum* and Asian congeners of this genus.

In this study, chromosomal morphology and behavior at various phases of the somatic cell cycle in *C. luteum* were examined by a conventional staining method. At the same time, the morphology, behavior, and properties as to the DNA base composition of heterochromatic segments were also investigated by fluorescent banding using probes specific for AT- or GC-rich DNA.

MATERIALS AND METHODS

Materials

A total of 12 plants, five from Alabama ('Al' hereafter) and four from West Virginia (WV), U.S.A., and three from an unknown source (Unk), were used for this study. These were obtained in 1979 to 2004 from the Far North Gardens, Michigan, U.S.A., or from two commercial nurseries in U.S.A. and Japan, and cultivated at the experimental nursery of Teikyo University at Hachioji, Tokyo. Cytological studies were performed mainly at the then botanical laboratory of the same university. Taxonomic identification of the plants followed Tanaka (2017).

Aceto-orcein staining

For observation of chromosome morphology and behavior at various phases of the cell cycle, excised root

tips approximately 1 to 1.5 mm long were fixed in 45% acetic acid for 7–10 min at 5–10°C, macerated in a 2 : 1 mixture of 1 M hydrochloric acid and 45% acetic acid for 30–50 s at 60°C, stained with 1 or 2 % aceto-orcein, and squashed after a coverslip was mounted on the sample. Some samples mounted with a coverslip were only gently tapped in order not to distort the nuclear or spindle structure. Some excised root tips were treated with 2 mM 8-hydroxyquinoline (8-HQ) for 3 or 4 h, sometimes for 6 h, at 18°C prior to fixation to observe its effects on chromosome morphology.

Data on chromosome arm ratios (long arm length / short arm length) were obtained for one metaphase spread (Fig. 1E) of Al-2 fixed directly. As the centromeres of *C. luteum* are relatively large, each arm length was expediently represented by the length from the midpoint of centromere to the end of arm. Terminology for centromeric position on chromosomes followed Levan *et al*. (1964).

Fluorescent chromosome banding

Excised root tips ca. 1.5–2 mm long were fixed in a 3 : 1 mixture of ethanol and acetic acid for 1 h at ca. 4°C, or treated with 2 mM 8-HQ for 3 or 4 h at 18°C prior to fixation in the same fixative, and rinsed in water at least for 15–20 min. They were macerated in an aqueous solution of 4% cellulase (Onozuka R-10, Yakult Co.) and 4% pectinase (Sigma), which was adjusted at pH 4.0–4.5 with 0.2 M HCl, for 1 h at 37–38°C, and rinsed in distilled water. A softened root tip was laid on a glass slide, and tissues surrounding the meristem as well as excessive water were removed. A small mass of the meristem on the slide was split into smaller pieces and evened with a dissecting needle tip under a stereomicroscope, and spread by adding one or two drops of the fixative onto the sample. The sample was flame-dried using a spirit lamp, and the slide was left at least overnight.

For fluorescent chromosome banding, schedules of several researchers such as Vosa (1970, 1971, 1973), Schweizer (1976, 1981), Schweizer and Nagl (1976) and Schnedl *et al*. (1980) were followed with modifications. Prior to staining with fluorochromes, some slides were treated with the following non-fluorescent antibiotics to see their effects on staining with fluorochromes; 0.2 mg/ml Actinomycin D (AMD; Sigma) in McIlvaine citric acid – Na_2HPO_4 buffer (McI.) at pH 7, or the same concentration of Distamycin A (DA; Sigma) in McI. at pH 7 for 10–15 min, and rinsed in the same buffer. Cell samples on slides were stained for 10–15 min with 0.5% quinacrine dihydrochloride (Q; Sigma) in distilled water, 0.5% quinacrine mustard dihydrochloride (QM; Sigma) in McI. at pH 7, 0.1 mg/ml chromomycin A_3 (CMA; Boehringer) or mithramycin (MM; Serva) in McI., containing 5–10 mM $MgCl_2$ at pH 7, 0.1 (sometimes 0.15 or 0.2) μg/ml 4′, 6-diamidino-2-phenylindol-dihydrochrolid (DAPI ; Boehringer), or Hoechst 33258

(H33258; Boehringer) in McI. at pH 7. After staining, excessive staining medium was removed by pouring the respective solvent over the sample. Samples stained with Q, QM, DAPI or H33258 were mounted in 50% glycerine (McI., pH 7), while those stained with CMA or MM were mounted in 50% glycerine (McI., pH 7) containing 5–10 mM $MgCl_2$. Of these dyes, Q, DAPI and CMA were frequently used compared with QM, H33258 and MM, for similar staining results were obtained respectively (i.e. Q vs. QM, DAPI vs. H33258, CMA vs. MM). Some stained slides were destained in 45% acetic acid for about 30 min or in the fixative for ca. 10 min, rinsed in water for 10–15 min, air-dried, and then restained with another fluorochrome prepared as noted above. In some cases, slides were sequentially double-stained, omitting destaining and air-drying, with fluorochromes exhibiting complementary base pair binding preferences; e.g. CMA as dye specific for GC pairs and DAPI as dye having high binding preference for AT pairs.

For observation of the stained samples, an Olympus microscope (BHB) equipped with an epi-illuminator was used. For samples stained with Q, QM, CMA or MM, one or two exciter filters BG 12, a dichroic mirror (DM 500) B, and a barrier filter (BF) O515 were used in combination to obtain optimum spectroscopical conditions for observation. For those stained with DAPI or H33258, one or two exciter filters UG1, a dichroic mirror (DM 400) U, and a barrier filter L420 were used in combination. Photomicrographs were taken on Kodak Tri-X or T-max panchromatic film.

RESULTS

Morphological and behavioral traits of somatic chromosomes during cell cycle - Results of aceto-orcein staining

In interphase nuclei, chromosomes formed 18 to 24 (21.4 on average; 20 nuclei examined of plant Al-2) comparatively large heteropycnotic segments (chromocenters) as well as faintly stained highly dispersed domains (Fig. 1A). The chromocenters were spheroidal, measuring ca. 0.6–1.1 μm in the long axis, and observed to be situated at the nuclear periphery in preparations only lightly tapped to avoid distorting nuclear or spindle structures. Even in squashed preparations, this trait was recognized for some chromocenters located at the inner periphery of flattened nuclei (Fig. 1A). Nuclei at interphase usually formed only one large nucleolus, but up to four nucleoli were detected in nuclei including telophase. A few (up to four) small condensed segments were often seen to be associated with nucleoli. It became evident from observations of cells at various phases that the chromocenters and the small condensed segments at interphase correspond respectively to the centromeric segments and the satellites on some chromosomes at
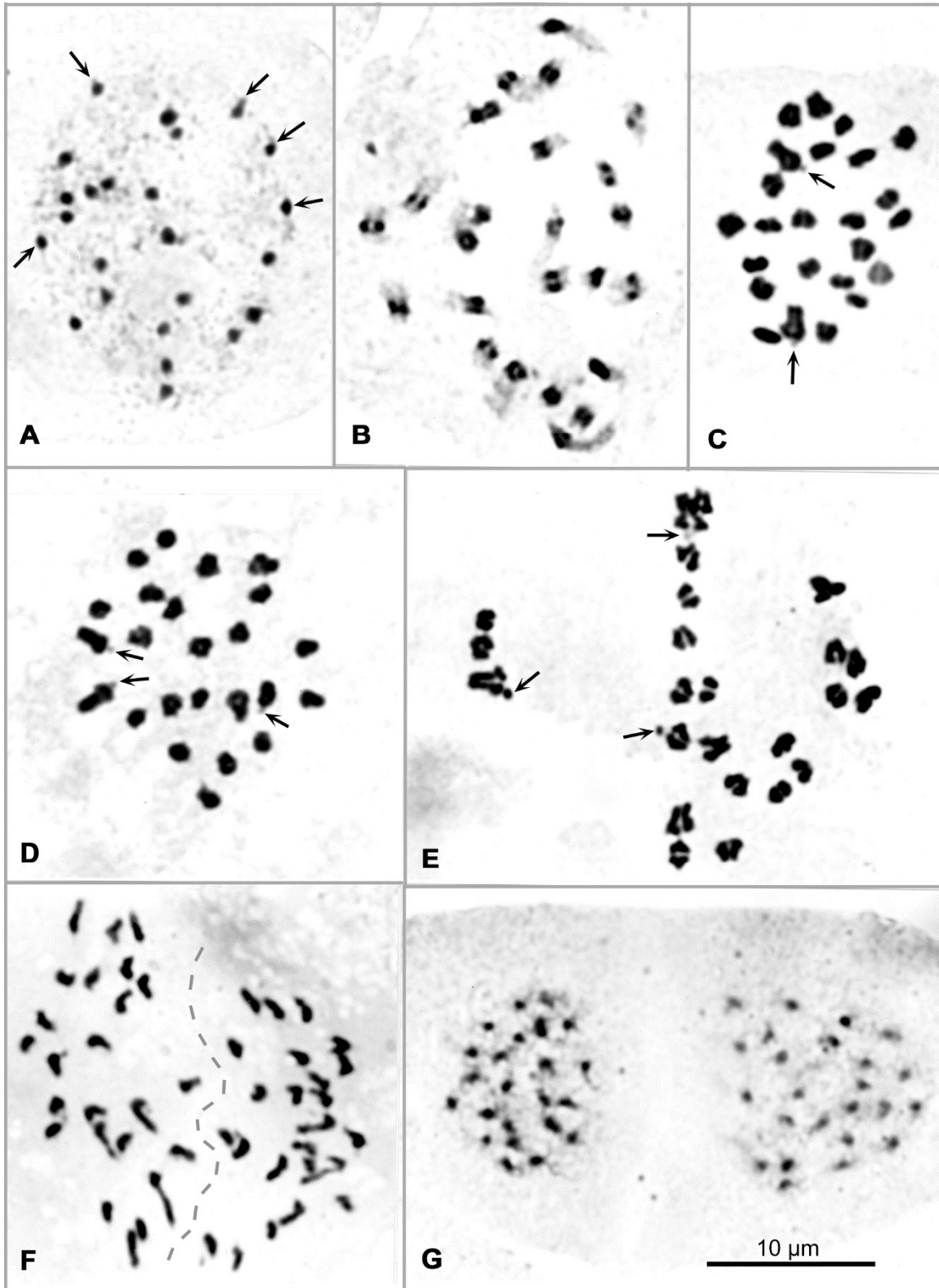
Fig. 1. Somatic chromosomes or nuclei of *C. luteum* (2*n* = 24) stained with aceto-orcein. **A**: Interphase nucleus with 24 chromocenters (Al-2, ♂), some located at inner periphery of nucleus arrowed. **B**: Prophase (Unk-1, ♂). **C–E**: Metaphase. **C** (Unk-3, sex unknown), **D** (WV-1, ♀), **E** (Al-2, ♂). Satellites seen are arrowed. **F**: Anaphase with two separating groups of sister chromatids (Unk-3, sex unkown). Dash line added to demarcate two groups. **G**: Telophase with two separated groups of sister chromatids (Unk-1, ♂). A, C: Pretreated with 8-HQ for 3 h. B, D–G: Fixed directly.
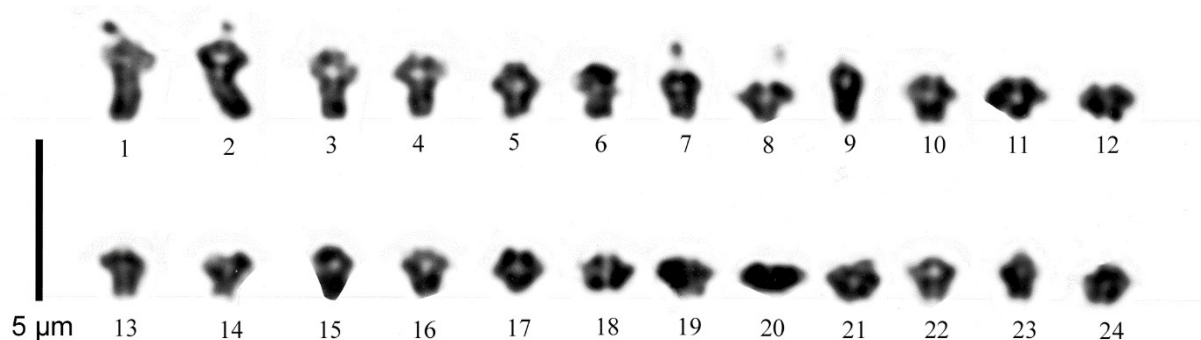
Fig. 2. Alignment of metaphase chromosomes of *C. luteum* (2*n* = 24; Unk-3, sex unknown) fixed without pretreatment with 8-HQ, and stained with aceto-orcein. In the text chromosomes 1 and 2 are designated as L, 3 and 4 as M, and the rest as S. Chromosomes 1, 2, 7 and 8 bear small satellites.

mitotic phases. In chromosomes at early to mid-prophase, the centromeric segments remained strongly condensed, while the chromosome arms were much less condensed, diffusing moderately toward distal ends (Fig. 1B). At late prophase the whole regions of chromosomes became more condensed, lying evenly scattered over the inner surface of the nucleus. During prometaphase the chromosomes migrated toward the center of a cell and eventually arranged themselves on the equatorial plane of the spindle. At metaphase, all of the 12 plants examined had 24 chromosomes (2*n* = 24), consisting of one pair of relatively long chromosomes (designated as L), one pair of long- or medium-sized chromosomes (M), and ten pairs of small chromosomes (S) (Figs. 1C–E, 2). The karyotype is therefore formulated as: 2*n* = 24 = 2L + 2M + 20S, although L, M and S were distinguished only by slight differences in length (Fig. 2). The longest pair (L) and one S or one pair of S usually had a small satellite (up to ca. 0.4 μm in length in L) at the distal end of the short arm respectively (Fig. 2). One plant (Unk-1) was observed to have only two satellites, each on one L and one S, whereas at least one other plant (Al-1) appears to have five small satellites on L and S (Fig. 3C, E). The satellites were not necessarily identical in size between homologous chromosomes (Fig. 1E). The numerical variation of the satellites among plants of this species is currently not fully grasped, hence needing further analyses. Chromosomes at metaphase did not form any primary constrictions, irrespective of the cells fixed directly (Figs. 1D, E, 2) or treated with 8-HQ prior to fixation (Fig. 1C). The centromeres of this species were located at the exact median point, median or submedian regions of chromosomes (arm ratios of chromosomes in one metaphase spread of Al-2 in Fig. 1E ranged 1.0–1.9 with the mean value 1.4), and tended to protrude in opposite directions (spindle poles). Lengths of the chromosomes including satellites in 10 metaphase spreads from two plants (Unk-1, -2) pretreated with 8-HQ for 3 h ranged from 1.0 (variation range: 0.8–1.4) to 2.2 (1.6–2.8) μm with the mean length 1.3 (1.1–1.7) μm, and the total chromosome lengths (2*n*) varied from 25.7 to 40.5 μm

with the mean length 32.1 μm. No concrete relationship between karyotype and sex of the plants was detected (Fig. 1D♀, E♂). At anaphase sister chromatids migrated, headed by their centromere regions, to spindle poles (Fig. 1F). At telophase centromeric regions remained compacted, and looked to be located at the periphery of the nucleus, whereas the chromosome arms were much loosened, diffusing toward the distal ends (Fig. 1G).

Heterochromatic segments visualized by fluorescent banding

The results of staining with DNA-binding fluorochromes and DNA-binding non-fluorescent antibiotics each with the known binding specificity indicated that there are two kinds of heterochromatin in *C. luteum*; one is of centromeres and the other is of satellites and their associated thin portions probably corresponding to nucleolus organizer regions (NORs). The centromeric heterochromatin fluoresced brightly with Q (Figs. 3A, 4B), QM, DAPI (Fig. 3B, D) or H33258, and its staining contrast was improved when prestained with AMD; i.e. AMD/Q (Figs. 4A, E) or AMD/DAPI (Fig. 4C, D). On the other hand, the heterochromatin of the satellites fluoresced brightly with CMA or MM, and its staining differentiation was enhanced when pretreated with DA; i.e. DA/CMA (Fig. 3C, E) or DA/MM. Metaphase chromosomes treated with DA in conjunction with DAPI (DA/DAPI) (Fig. 4F) or Q (DA/Q) resulted in producing very dim, almost uniform fluorescence including centromeric segments and satellites (Table 1).

Centromeric heterochromatin

The centromeric segments fluoresced brightly not only with (AMD/)Q (Figs. 3A, 4A, B, E) and QM, but also with (AMD/)DAPI (Figs. 3B, D, 4C, D) or (AMD/)H33258, as noted above. The correspondence between the segments stained with the former or the latter group of drugs was ascertained by examining the same preparations stained first with (AMD/)Q (Fig. 3A), and then destained and restained with (AMD/)DAPI (Fig. 3B). When treated with (DA/)CMA or (DA/)MM, the

Table 1. Staining reactions of heterochromatic segments in *C. luteum* to some DNA ligands used.

Heterochromatin	Q, AMD/Q	QM	DAPI, AMD/DAPI	Hoechst 33258	CMA, DA/CMA	MM, DA/MM	DA/Q	DA/DAPI
Centromere	+	+	+	+	-	-	0	0
Satellite (NOR)	-	-	-	-	+	+	0	0

Symbols.   + : positive.   - : negative.   0 : neutral (undifferentiated from euchromatic regions).
For abbreviations of drugs used, see text.

centromeric segments were unstained (Fig. 3C, E). The correspondence between the segments was likewise ascertained in the same preparations stained first with (DA/)CMA (Figs. 3C, E) and then restained with (AMD/)Q or (AMD/)DAPI (Fig. 3D, F).

At prophase the sister centromeric segments were juxtaposed as close as the arm regions (Fig. 3A, B, D). At metaphase they were nearly as thick as the arms (Fig. 4A) and tended to lie on lateral sides of sister chromatids, protruding in opposite directions, irrespective of the cells fixed directly (Fig. 4B) or treated with 8-HQ prior to fixation (Fig. 4A, C). At anaphase they moved, leading their arms, toward spindle poles (Fig. 4D). At telophase the segments remained highly compacted, being ca. 0.5 to 1.0 μm in the major axis, while the chromosome arms became fairly unraveled, dispersing toward distal ends (Fig. 4E). The chromocenters in interphase nuclei (Fig. 3F) were spheroidal and ca. 0.5 to 1.5 μm in the long axis. Some or many of them at interphase were often somewhat larger than those at telophase (Fig. 4E), probably indicating that they were replicated.

Heterochromatin of satellites

In mitotic chromosomes and interphase nuclei, there were segments fluorescing brightly with (DA/)CMA (Fig. 3C, E) or (DA/)MM. They were small, counting two, four or five (Fig. 3C, E) depending on plants examined. They were unstained or vaguely stained with (AMD/)Q or (AMD/)DAPI (Fig. 3D, F; Table 1).

Pericentric euchromatic regions

When treated with (DA/)CMA or (DA/)MM, the pericentric regions of all chromosomes at prophase were stained brighter than the distal part of the arms (Fig. 3C), probably reflecting their stronger condensation. This staining trait was, however, indistinct when stained with (AMD/)Q (Fig. 3A) or (AMD/)DAPI (Fig. 3B, D).

DISCUSSION

The comparatively large spheroidal heterochromatic segments at metaphase of *C. luteum* tended to lie on lateral sides of sister chromatids (Fig. 4B, C), protruding in opposite directions (Figs. 1C–E, 2, 4B, C, F). At anaphase they migrated, leading the arms, toward spindle poles (Figs. 1F, 4D). These observations signify that the segments are pulled by a number of spindle fibers, and accordingly, the segments can be interpreted as centromeres themselves. While the centromeres of *C. luteum* are similar to ordinary monocentromeres in being

localized to one portion of each chromosome (Figs. 1B–F, 2, 3A–D, 4A–D), they differ from the latter in magnitude and in not forming constrictions at metaphase (Figs. 1C–E, 2, 4A–C, F). On the other hand, they look somewhat similar to holocentromeres in being extended, though in a limited manner, along lateral poleward sides of chromosomes (e.g. Fig. 4A–C). The centromeres of *C. luteum* thus appear different from both types of centromere so far known, and as a novel type of centromere they are provisionally termed here 'macrocentromeres'. As the chromosomes of some Asian congeners are reported to be holocentric, the macrocentromeres of *C. luteum* possibly represent a precursory state from which holocentromeres once evolved in an ancestral lineage of the Asian species.

Fluorochromes Q, QM, DAPI and H33258, and a non-fluorescent antibiotic DA are reported to have high binding specificity for AT base pairs (Weisblum, 1973; Weisblum and de Haseth, 1973; Weisblum and Haenssler, 1974; Comings, 1975; Comings and Drets, 1976; Schweizer, 1976, 1981; Lin *et al*., 1977; Schlammadinger *et al*., 1977; Mezzanotte *et al*., 1979; Schnedl *et al*., 1980). On the other hand, fluorochromes CMA and MM and a non-fluorescent antibiotic AMD are reported to have high binding specificity for GC base pairs (Behr *et al*., 1969; Schweizer, 1976, 1981). Judging from the staining reactions to all these drugs (Table 1), it is evident that the centromeric heterochromatin of *C. luteum* is rich in AT base pairs.

Observations on the morphology, spatial location and number of chromocenters in interphase nuclei suggest that the centromeric domains, which correspond exactly to chromocenters, are tightly compacted almost through interphase, lying at the nuclear periphery (Fig. 1A). This implies that centromeres have two major roles; one is, as is well known, as loci for transmitting chromosomes to subsequent generations through mitoses and meioses. The other is as loci for anchoring chromosomes to the inner surface of the nucleus during telophase, interphase and prophase. Observations similar to this have been reported for many other higher plants; in them chromosomes largely retain their relative spatial disposition or domains during telophase through interphase to prophase with their centromere regions anchored to the inner surface of the nucleus (Tanaka and Tanaka, 1977b; Tanaka, 1981a, b, c). Using a four-dimensional live cell imaging technique, Fang and Spector (2005) also reported that centromeres of *Arabidopsis* are constrained at the nuclear periphery during interphase. Considering that the centromeric
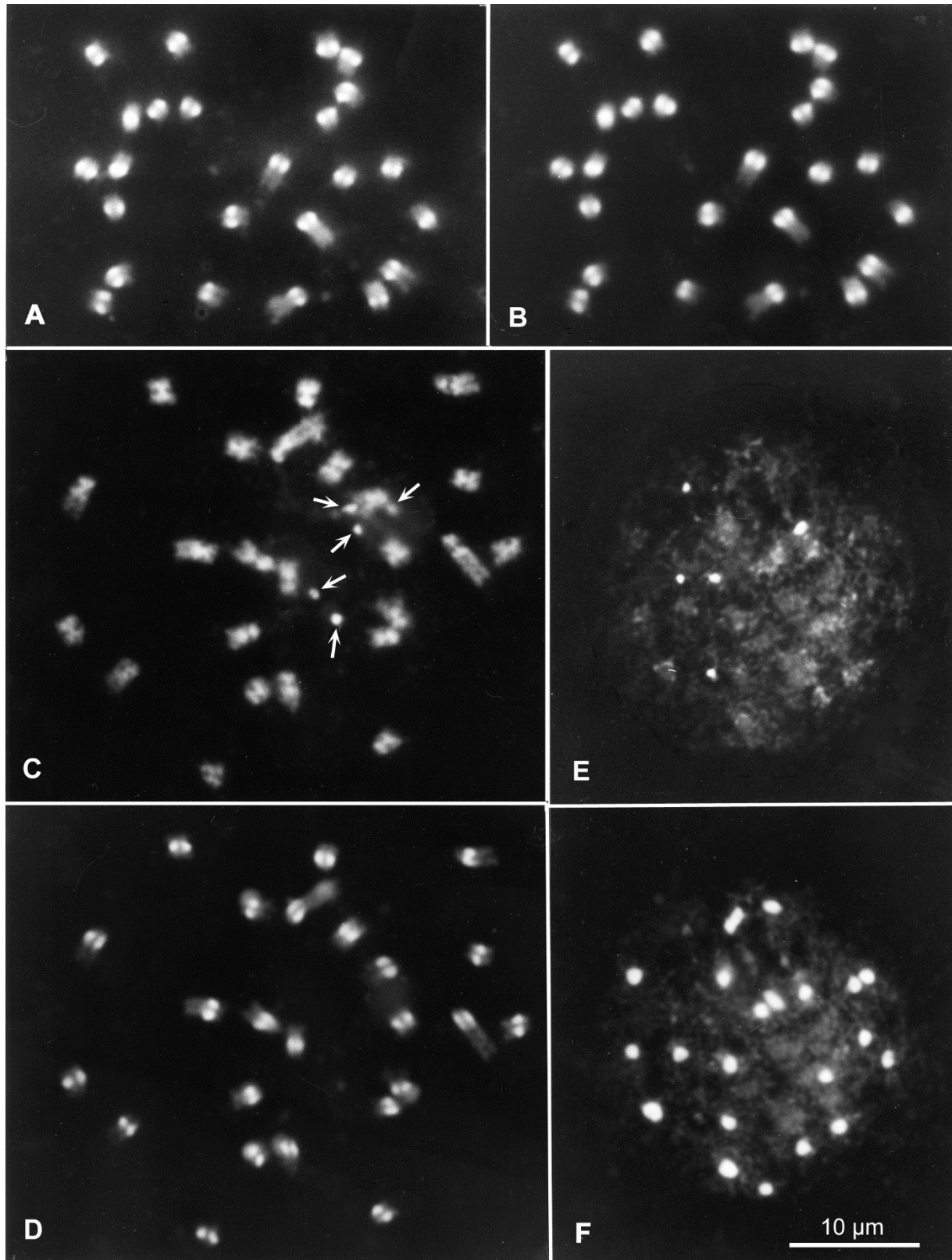
Fig. 3. Fluorescent banding of somatic chromosomes or nuclei of *C. luteum* (2*n* = 24). **A–D**: Prophase. **A**: Stained with Q (Al-2, ♂). **B**: Same cell as A; restained with DAPI after destaining Q. **C**: Stained with DA/CMA (Al-1, sex unknown). Five small satellites arrowed. **D**: Same cell as C; restained with DAPI after staining with DA/CMA. **E**: Interphase nucleus stained with DA/CMA (Al-1, sex unknown). Five satellites stained brightly. **F**: Same cell as E; restained with DAPI after staining with DA/CMA. All cells pretreated with 8-HQ for 3 h.
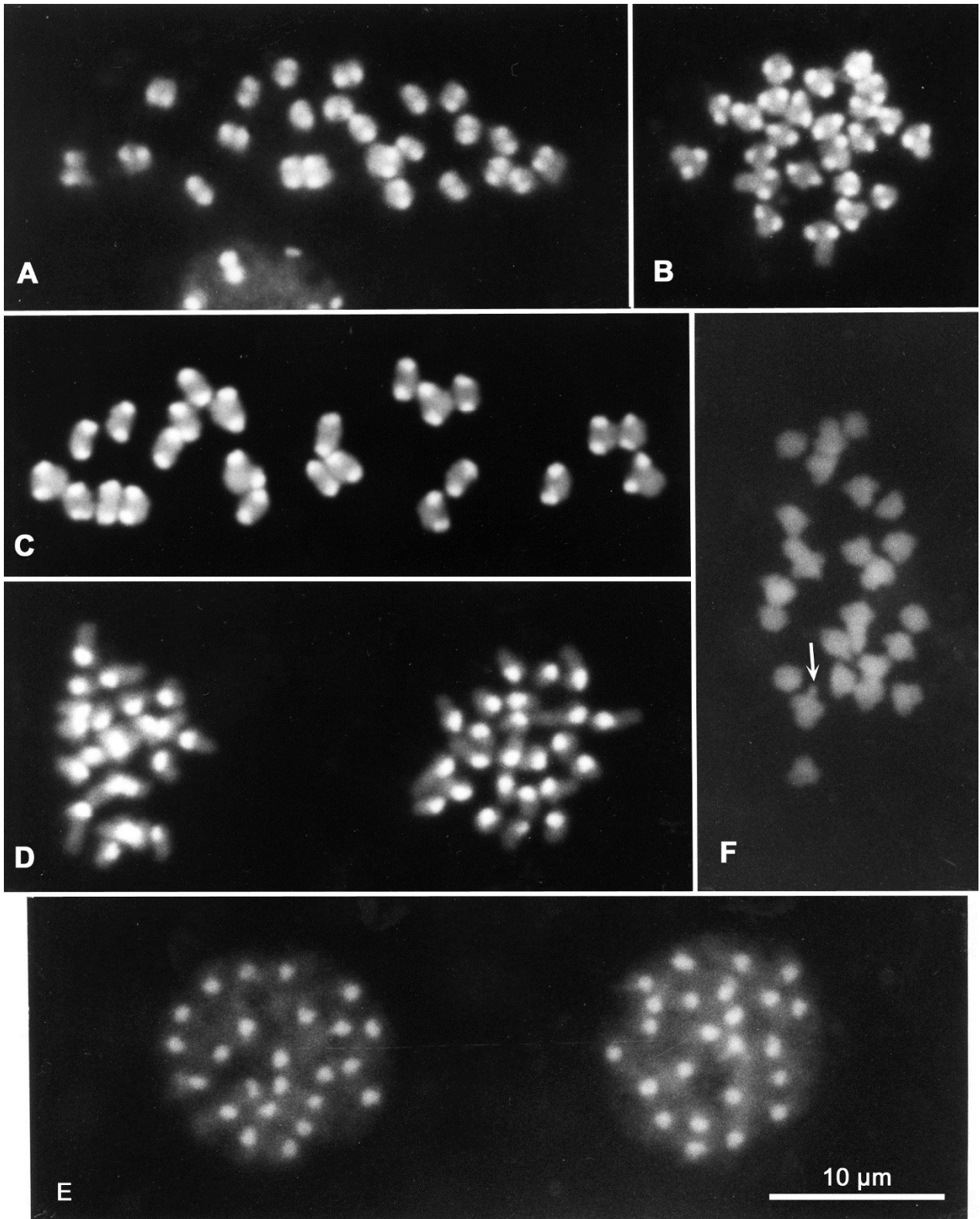
Fig. 4. Fluorescent banding of somatic chromosomes or nuclei of *C. luteum* ($2n = 24$). **A–C, F**: Metaphase. **A**: Stained with AMD/Q (Unk-1, ♂). **B**: Stained with Q (Al-2, ♂). **C**. Stained first with AMD/Q, then destained and restained with AMD/DAPI (Al-2, ♂). **D**: Two separating groups of sister chromatids at anaphase, stained first with AMD/Q, then destained and restained with AMD/DAPI (Al-2, ♂). **E**: Two daughter nuclei at telophase, stained with AMD/Q (Al-2, ♂). **F**: Stained with DA/DAPI (Unk-1, ♂). One satellite on small chromosome (S) is arrowed. A: Pretreated with 8-HQ for 4 h. C: Pretreated with 8-HQ for 3 h. The other cells fixed directly.

heterochromatin of *C. luteum* is constituted of copious amounts of AT-rich DNA and tightly compacted almost throughout the cell cycle, it is highly likely that the centromeric DNA is transcriptionally scarcely active and serves as a component for structuring centromeres. Owing to their roles to play, centromeres are perhaps suitable loci for various non-coding sequences to harbor. This view accords with the least occurrence of coding DNA in centromeres and with inconsistent, non-conservative base composition of centromeric DNA reported for various organisms (Houben and Schubert 2003; Nagaki *et al*. 2005; Nagaki 2009; Demidov *et al*. 2014; Plohl *et al*. 2014; Cuacos *et al*., 2015).

As in other higher organisms, euchromatic chromosome arms of *C. luteum* exhibited a regular, polarized condensation/decondensation pattern during mitotic phases; i.e. centripetal condensation mainly at prophase (Figs. 1B, 3A–D) and centrifugal decondensation at telophase (Figs. 1G, 4E). It is again highly likely that centromeres play a role of anchors for such regular, polarized cyclic structural alterations. By attaching to the nuclear envelope, centromeres may serve as anchors for securing all orderly structural and functional activities of chromosomes within the nucleus.

Often seen to be associated with nucleoli, the satellites and associated thin portions of *C. luteum* may be regarded as NORs (Fig. 2). Judging from the staining reactions to the drugs used (Table 1), the NORs of *C. lutem* are rich in GC base pairs, coinciding with cases in many other species (Schweizer, 1976; 1981, Schmid; 1980; Hizume *et al*., 1988a, b; Okada, 1991; Guerra, 2000).

Chromosomes of *C. luteum* are monocentric. In contrast, those of three Asian species of this genus (subsect. *Chionographis*) are reported to be holocentric. Besides the difference in centromere organization, these species also differ in the following respects. The number of chromocenters in an interphase nucleus of *C. luteum* is usually a few less than the somatic chromosome number (the average number of chromocenters per nucleus was 21.4, which is 89.2 % of the maximum number 24) (Figs. 1A, 3F), whereas heteropycnotic segments found in interphase nuclei of *C. japonicum* are more numerous, exceeding the somatic chromosome number 24 and often smaller (Tanaka and Tanaka, 1979; Tanaka, 2020a). This difference is likely to stem from the difference in centromere organization of the two species, for chromocenters of *C. luteum* are actually centromeres. In *C. luteum*, satellites and secondary constrictions are formed (e.g. Figs. 1C–E, 2, 3C). In contrast, they are not detected in the Asian congeners (Tanaka and Tanaka, 1979, Tanaka, 2020a, b), although at least one pachynema was observed to be attached to a nucleolus at meiotic prophase (Tanaka and Tanaka, 1980). According to Pellicer *et al*. (2014), who employed flow cytometry for assessing nuclear DNA amount, *C. luteum* showed a significantly smaller value

than *C. japonicum* (0.98 vs. 1.53 pg for 1C). A further critical comparison of these highly contrastive chromosomes in *C. luteum* and Asian species of this genus may shed light on how and to what degree their chromosomes became differentiated in the course of evolution.

ACKNOWLEDGMENTS

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