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Molecular-cytotaxonomy of *Cycas* (Cycadales) Using 5S Ribosomal DNA Markers

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Abstract Somatic chromosomes of fourteen species of *Cycas* (Cycadaceae, Cycadales; Stevenson, 1992) were compared by the fluorescence *in situ* hybridization (FISH) method using 5S ribosomal DNA (5S rDNA) probes. The fourteen species of *Cycas* showed the same chromosome number of 2n=22, and a similar karyotype consisting of 4 st, 12 t, 4 sm and 2 m chromosomes. In the FISH comparison, *C. revoluta*, *C. taitungensis*, *C. wadei*, *C. diannanensis* and *C. micholitzii* exhibited a 5S rDNA site on two t chromosomes in a metaphase complement. On the other hand, *C. siamensis*, *C. circinalis*, *C. cairnsiana*, *C. couttsiana*, *C. media*, *C. megacarpa*, *C. micronesica* and *C. thouarsii* exhibited a 5S rDNA site on four t chromosomes in a metaphase complement. The two cytological groups based on the number of 5S rDNA agreed with the taxonomic treatment and phylogenetic hypothesis suggested by Hill (1996, 2004), but disagreed with those by De Laubenfels and Adema (1998). Recognition of the genus *Epicycas* as discrete from *Cycas* is not justified on the basis of cytology.

Key words: 5S rDNA, *Cycas*, cytotaxonomy, FISH.

The genus *Cycas* (Cycadaceae, Cycadales; Stevenson, 1992) primarily occurs in Australia and the eastern parts of mainland Asia, with small numbers of species extending to Japan, Melanesia, Micronesia, Polynesia and Africa. This genus is the most widely distributed and the largest in the Cycadales (Stevenson *et al.*, 1995; Hill *et al.*, 2004). Although taxonomical studies of *Cycas sensu lato* based on morphological comparisons have been advancing (Hill, 1992, 1994a, 1994b, 1995, 1996, 2004; De Laubenfels and Adema, 1998; Chen *et al.*, 2004), cytological studies have been poorly published on this genus.

Recently the molecular-cytological technique of the FISH method has been applied to cytotaxonomic comparisons in certain Cycadales taxa, and it has been shown to be a powerful method for analyzing intergeneric- and infrageneric-chromosomal relationships in Zamiaceae (Kokubugata and Kondo, 1998; Kondo and Tagashira, 1998; Tagashira and Kondo, 2001), *Bowenia* and *Stangeria* (Kokubugata *et al.*, 2000a; Kokubugata *et al.*, 2002b) and in *Cycas* (Kokubugata *et al.* 2002b).

The specific aims of the present study are to compare the 5S rDNA sites on somatic chromosomes of fourteen *Cycas* species by the FISH method for considering interspecific-taxonomical relationships in this genus. In particular, the cytological characteristics of species referred to the

genus *Epicycas* by De Laubenfels and Adema (1998) are compared to those that they left in the genus *Cycas*.

Materials and Methods

Plant Materials

In the present study, we investigated chromosomes of six species of *Cycas sensu lato*, *C. diannanensis* Z. T. Guan et G. D. Tao (=*Epicycas diannanensis* (Z. T. Guan et G. D. Tao) De Laubenfels), *C. micholitzii* Dyer (=*E. micholitzii* (Dyer) De Laubenfels), *C. cairnsiana* F. Muell, *C. couttsiana* K. D. Hill, *C. megacarpa* K. D. Hill and *C. ophiolitica* K. D. Hill by the fluorescence *in situ* hybridization (FISH) using 5S ribosomal DNA (5S rDNA) probes (Table 1). The 5S rDNA distribution patterns on somatic chromosomes of the six species were compared with those of eight species previously examined by Kokubugata *et al.* (2002b, Table 1), namely *C. revoluta* Thunb., *C. taitungensis* C. F. Shen, K. D. Hill, C. H. Tsou et C. J. Chen, *C. wadei* Merr., *C. siamensis* Miq. (=*E. siamensis* (Miq.) De Laubenfels), *C. circinalis* L., *C. media* R. Br., *C. micronesica* K. D. Hill and *C. thouarsii* R. Br.

Plants of six *Cycas* species cultivated in the Tsukuba Botanical Garden were investigated in the present study (Table 1). Young leaflets of the cultivated plants were collected and pretreated in 2 mM 8-hydroxyquinoline at 4°C for 20 h, and fixed in acetic ethanol (1 : 3) at 4°C for 24 h then stored in 70% ethanol under 0°C for the present FISH experiments.

Probes and Labeling of PCR-amplified 5S rDNA for FISH

5S rDNA sequences were amplified by total genomic DNA of *C. revoluta* cultivated in the Tsukuba Botanical Garden (accession no. TBG 140356) by the polymerase chain reaction (PCR)

Table 1. Fourteen species *Cycas sensu lato* and their cytological characters compared in the present study.

De Laubenfels & Adema (1998)	Hill (1996, 2004)		Distribution	2n	Karyotype	No. 5S rDNA	Reference*
	Species	Section					
Cycas revoluta	*Cycas revoluta*	*Asiorientales*	Japan	22	12 t+4 st+4 sm+2 m	2	a
Cycas taitungensis	*Cycas taitungensis*	*Asiorientales*	Taiwan	22	12 t+4 st+4 sm+2 m	2	a
Cycas wadei	*Cycas wadei*	*Wadeanae*	Philippines	22	12 t+4 st+4 sm+2 m	2	a
Epicycas diannanensis	*Cycas diannanensis*	*Stangerioides*	China, Laos, Vietnam	22	12 t+4 st+4 sm+2 m	2	b
Epicycas micholitzii	*Cycas micholitzii*	*Stangerioides*	China, Laos, Vietnam	22	12 t+4 st+4 sm+2 m	4	b
Epicycas siamensis	*Cycas siamensis*	*Indosinenses*	Thailand	22	12 t+4 st+4 sm+2 m	4	a
Cycas cairnsiana	*Cycas cairnsiana*	*Cycas*	Australia	22	12 t+4 st+4 sm+2 m	4	b
Cycas circinalis	*Cycas circinalis*	*Cycas*	India	22	12 t+4 st+4 sm+2 m	4	a
Cycas couttsiana	*Cycas couttsiana*	*Cycas*	Australia	22	12 t+4 st+4 sm+2 m	4	b
Cycas media	*Cycas media*	*Cycas*	Australia	22	12 t+4 st+4 sm+2 m	4	a
Cycas megacarpa	*Cycas megacarpa*	*Cycas*	Australia	22	12 t+4 st+4 sm+2 m	4	b
Cycas micronesica	*Cycas micronesica*	*Cycas*	Guam	22	12 t+4 st+4 sm+2 m	4	a
Cycas ophiolitica	*Cycas ophiolitica*	*Cycas*	Australia	22	12 t+4 st+4 sm+2 m	4	b
Cycas thouarsii	*Cycas thouarsii*	*Cycas*	Madagascar	22	12 t+4 st+4 sm+2 m	4	a

*a. Kokubugata *et al.* (2002b). b. the present study.

following Hizume (1995). The PCR-amplified 5S rDNA was labeled with digoxigenin-(DIG-)14-dATP by the nick translation method. The labeled probe was dissolved in 50% formamide and 10% dextran sulfate (w/v) in 2X SSC. This hybridization mixture was denatured at 75°C for 10 min, then immediately chilled in cold water for 10 min and stored at 0°C.

Fluorescent *in situ* Hybridization (FISH)

The stored leaflets in 70% ethanol were macerated in an enzyme solution with 2% cellulase "Onozuka" RS (Yakult) and 1% pectoryase "Y-23" (Seisin) in distilled water (w/v; pH 4.5) at 36°C for 20 min before being washed in distilled water and 45% acetic acid at room temperature for 5 min. The macerated leaflet was placed on a slide and squashed in 45% acetic acid with a cover slip by the standard squash method. The slide was dried at 36°C for 30 min after removing the cover slip with a dry-ice block. It was digested with 0.1% RNase in 2X SSC at 36°C for 1 h, washed in 2X SSC for 10 min, treated in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min and washed in distilled water for 10 min. The slide was then dehydrated in 75%, 85% and 100% ethanol for 3 min each before being dried at 36°C for 20 min. The dried slide received 10 μl of the hybridization mixture and was covered with a cover slip and sealed with rubber cement. The probe DNA and chromosomal DNA on the slide were then denatured at 75°C for 15 min on a hot plate and immediately incubated at 37°C in a humid chamber overnight for DNA hybridization to occur. Following the hybridization, the cover slip was removed and the slide was twice washed in 4X SSC at 40°C for 10 min. The hybridized probes on chromosomal DNA were detected with 20 μg/ml Anti-digoxigenin-fluorescein, Fab-fragmentavidin in 1% bovine serum albumin in 4X SSC at 37°C for 1 h. The slide was twice washed in 4X SSC at room temperature for 10 min before receiving 30 μl of a mixed solution of 10% PBS, 90% glycerol with 0.1g/ml 1,4-Diazabicyclo [2.2.2.] octan and 1 μg/ml propidium iodide (PI). The slide was then mounted with a cover slip for counter staining and held at 4°C for 2 h at least.

The hybridization signals were made visible by Anti-digoxigenin-fluorescein, Fab-fragmentavidin as yellow fluorescence using the single band pass excitation filter (Zeizz filter set no. 2). Non hybridized regions were made visible by PI as reddish fluorescence using the single band pass excitation filter (Zeizz filter set no. 15). The two fluorescences were respectively captured by a Pixera Penguin 600 CL (DiRactor^{TM}) camera as digital images. The two digital images were overlapped after being modified to the best contrast between signal and PI-counterstain using the software of Lumina Vision OL (Mitani), and then the overlapped-color image was converted to a black and white image. The signals were visible as lighter bands or dots in the black and white images.

Description of Chromosomes

The chromosomes at mitotic metaphase were classified by arm ratio (R＝long arm length/short arm length) following Levan *et al.* (1964). Median-centromeric (1.0≦R＜1.7), submedian-centromeric (1.8≦R＜3.0), subterminal-centromeric (3.1≦R＜7.0), and terminal-centromeric chromosomes (7.1≦R) are respectively abbreviated as m, sm, st and t chromosomes in this paper.

Results and Discussion

Chromosome Number and Karyotype

Six species of *Cycas sensu lato* (*s. l.*), namely *C. diannanensis* (＝*E. diannanensis*), *C. mi-*

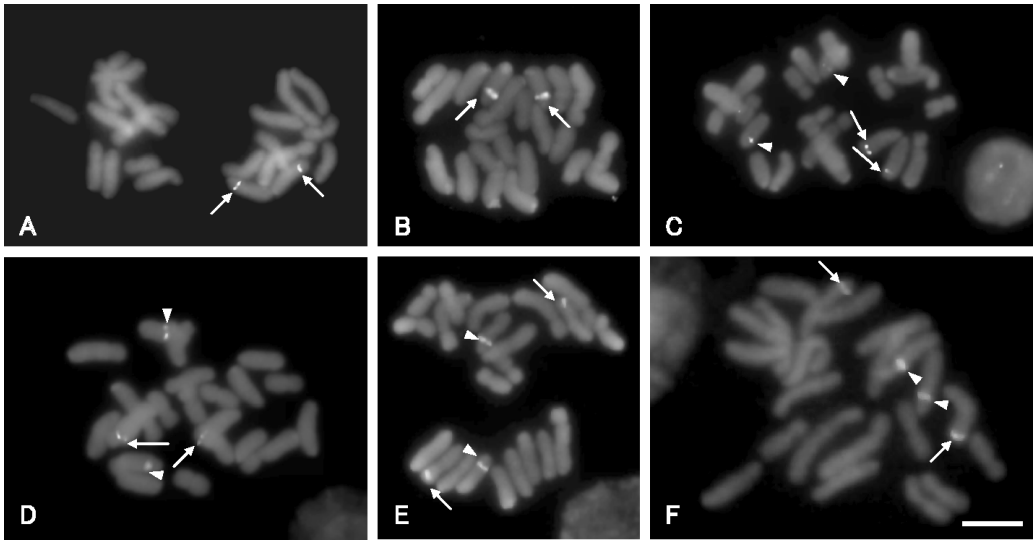

Fig. 1. FISH detected chromosomes of six species of *Cycas sensu lato* investigated in the present study. A. *C. diannanensis* (=*Epicycas diannanensis*). B. *C. micholitzii* (=*E. micholitzii*), C. *C. cairnsiana*. D. *C. couttsiana*. E. *C. megacarpa*. F. *C. ophiolitica*. Lighter fluorescences indicated by allows are 5S rDNA sites. Scale bar indicates 10 μm.

cholitzii (=*E. micholitzii*), *C. cairnsiana*, *C. couttsiana*, *C. megacarpa* and *C. ophiolitica* observed in the present study showed the same chromosome number of 2n=22. The chromosome number for *C. ophiolitica* confirmed that previously reported by Kokubugata *et al.* (2002c), while those of *C. diannanensis* (=*E. diannanensis*), *C. micholitzii* (=*E. micholitzii*), *C. cairnsiana*, *C. couttsiana* and *C. megacarpa* are reported for the first time in the present study. The chromosome numbers of the six species examined in the present study corresponded with those of eight species of *Cycas s. l.* previously reported, namely *C. revoluta*, *C. taitungensis*, *C. wadei*, *C. siamensis* (=*E. siamensis*), *C. circinalis*, *C. media*, *C. micronesica* and *C. thouarsii* (e.g. Ishikawa, 1916; Ohri and Khoshoo, 1986; Peng *et al.*, 1986; Paiva and Leitao, 1987; Kondo *et al.*, 1995; Kokubugata *et al.*, 2000b, 2002b, 2002c).

At mitotic metaphase karyotypes of the six species of *Cycas s. l.* consisted of four long st, twelve t, four short sm (or m) and two short m chromosomes. The karyotypes of the six species were similar to those of the other eight species previously reported (Hizume *et al.*, 1992; Kokubugata and Kondo, 1994; Hizume, 1995; Kondo *et al.*, 1995; Hizume *et al.*, 1998; Kokubugata *et al.*, 2000b, 2002b, 2002c).

In the present study *C. diannanensis* (=*E. diannanensis*) and *C. micholitzii* (=*E. micholitzii*) exhibited a 5S rDNA site on the interstitial region near the terminal of two t chromosomes (Figs. 1A, B & 2D, E). On the other hand, *C. cairnsiana*, *C. couttsiana*, *C. megacarpa* and *C. ophiolitica* exhibited a 5S rDNA site on the interstitial region near the terminal in two t chromosomes and on the internal region near the centromere of the other two t chromosomes (Figs. 1C, D, E, F & 2G, I, K, M).

Previously Kokubugata *et al.* (2002b) reported *C. revoluta*, *C. taitungensis*, *C. wadei* as exhibiting a 5S rDNA site on the interstitial region near the terminal of two t chromosomes (Fig. 2A, B, C) similar to *C. diannanensis* (=*E. diannanensis*) and *C. micholitzii* (=*E. micholitzii*) (Fig. 2D, E). They also reported *C. siamensis* (=*E. siamensis*), *C. circinalis*, *C. media*, *C. mi-*

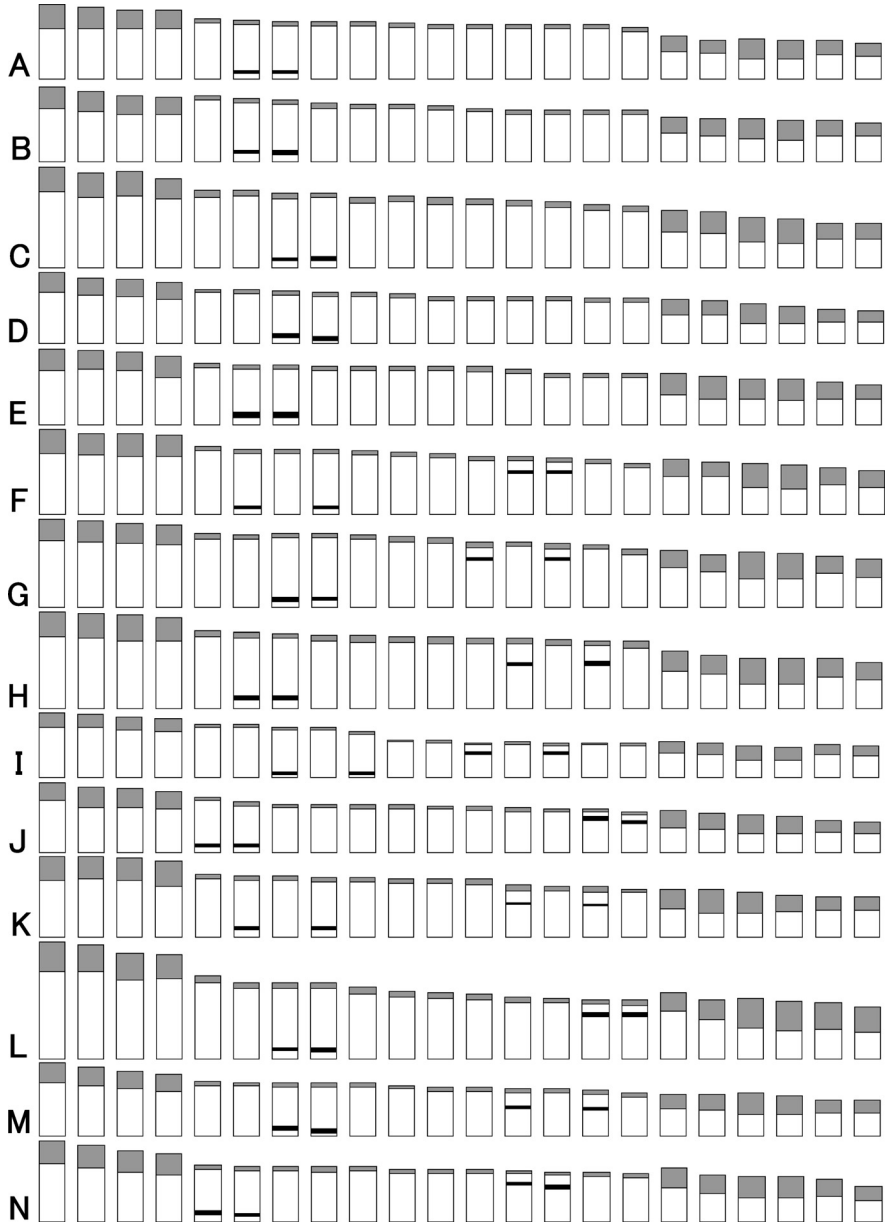

Fig. 2. Ideograms of fourteen species of *Cycas sensu lato* compared in the present study. A. *C. revoluta*. B. *C. taitungensis*. C. *C. wadei*. D. *C. diannanensis* (=*Epicycas diannanensis*). E. *C. micholitzii* (=*E. micholitzii*). F. *C. siamensis* (=*E. siamensis*). G. *C. cairnsiana*. H. *C. circinalis*. I. *Cycas* couttsiana. J. *C. media*. K. *C. megacarpa*. L. *C. micronesica*. M. *C. ophiolitica*. N. *C. thouarsii*. Opened areas indicated long arms. Grayish areas indicated short arms. Solid bands indicated 5S rDNA sites.

cronesica and *C. thouarsii* as exhibiting a 5S rDNA site on the interstitial region near the terminal in two t chromosomes and on the internal region near the centromere of the other two t chromosomes (Fig. 2F, H, J, L, N) similar to *C. cairnsiana*, *C. couttsiana*, *C. megacarpa* and *C. ophiolitica*.

Taxonomic Background

Hill (1996, 2004) suggested a classification of genus *Cycas* into five sections based on morphological characters. According to Hill (1996, 2004), *C. revoluta* and *C. taitungensis* were classified into section *Asiorientales* with another Chinese species *viz*. *C. panzihuaensis* L. Zhou & S. Y. Yang; *C. wadei* was classified into section Wadeanae with another Philippine species *viz*. *C. curranii* (Schuster) K. D. Hill; *C. diannanensis* and *C. micholitzii* were classified into section *Stangerioides* with another 26 species occurring from China to India; *C. siamensis* was classified into section *Indosinenses* with thirteen other species occurring from China to India; and *C. circinalis*, *C. cairnsiana*, *C. couttsiana*, *C. media*, *C. megacarpa*, *C. micronesica*, *C. ophiolitica* and *C. thouarsii* were classified into section *Cycas* with 46 species occurring in Australia, Melanesia, Micronesia, Polynesia and Africa. Moreover, Hill (2004) recognized that there were two major phylogenetic groups in the genus *Cycas* based on molecular and morphological characters: one consisted of sections *Asiorientales*, *Wadeanae* and *Stangerioides*, and the other consisted of sections *Indosinenses* and *Cycas*.

On the other hand, De Laubenfels and Adema (1998) described a new genus *Epicycas* De Laubenfels with the type of *Epicycas micholitzii* (Dyer) De Laubenfels. They distinguished the two genera *Cycas* and *Epicycas* on the basis of the former with well developed trunks and the latter with underground trunks. According to De Laubenfels and Adema (1998), *C. diannanensis*, *C. multipinnata* and *C. siamensis sensu* Hill (2004) would be classified into the genus *Epicycas* and respectively treated as *E. diannanensis*, *E. multipinnata* and *E. siamensis*. The other eleven species would remain classified into the genus *Cycas*. However, Chen *et al.* (2004) suggested that genus *Epicycas* was not a natural group based on morphological characters and the genus has not gained taxonomic recognition.

Taxonomic Conclusions

In the present FISH comparison, the fourteen species can be classified into two cytological groups: one is characterized by two 5S rDNA sites in a mitotic complement, and consists of *C. revoluta*, *C. taitungensis*, *C. wadei*, *C. diannanensis* (=*E. diannanensis*) and *C. micholitzii* (=*E. micholitzii*); the other is characterized by four 5S rDNA sites in a mitotic complement and consists of *C. siamensis* (=*E. siamensis*), *C. circinalis*, *C. cairnsiana*, *C. couttsiana*, *C. media*, *C. megacarpa*, *C. micronesica*, *C. ophiolitica* and *C. thouarsii*. The two cytological groups based on the number of the 5S rDNA site agree with the two phylogenetic groups based on morphological and molecular data suggested by Hill (2004). The two cytological groups do not support the recognition of two genera suggested by De Laubenfels and Adema (1998) because *C. diannanensis* (=*E. diannanensis*) and *C. multipinnata* (=*E. multipinnata*) are cytologically close to *C. revoluta*, *C. taitungensis* and *C. wadei*; and *C. siamensis* (=*E. siamensis*) is cytologically close to *C. circinalis*, *C. cairnsiana*, *C. couttsiana*, *C. media*, *C. megacarpa*, *C. micronesica*, *C. ophiolitica* and *C. thouarsii*.

In conclusion, the present FISH study does not support the taxonomic treatment of De Laubenfels and Adema (1998), but does support the phylogenetic hypothesis of Hill (1996, 2004).

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