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Inter- and intraspecific variations of chloroplast DNA of the siphonous green algal genus *Caulerpa* (Caulerpales, Chlorophyta)

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Chloroplast DNA (cpDNA) from four species of the siphonous green algal genus *Caulerpa*, *C. lentillifera*, *C. okamurae*, *C. racemosa* var. *clavifera* f. *macrophysa*, and *C. brachypus* were isolated and analyzed by restriction endonucleases. The restriction patterns were extremely heterogeneous, but a distinct similarity was found between *C. lentillifera* and *C. okamurae*. The genome sizes of cpDNA of *C. lentillifera* and *C. okamurae* were calculated to be about 90 kb, and the genome sizes of cpDNA of *C. racemosa* and *C. brachypus* were more than 100 kb. These results were compared and discussed in relation to their morphological characters.

In addition, the cpDNAs of *C. racemosa* collected from two areas in Okinawa Island were compared. The restriction patterns were almost the same, but a few changes were detected.

Key Index Words: Caulerpa—*chloroplast DNA—green algae—restriction analysis —RFLP.*

The siphonous green algal genus *Caulerpa* is a large group including 73 species and 111 infraspecific taxa (Calvert *et al.* 1976). The classification of this genus at the species level is mainly according to morphological characteristics of the vegetative thallus and the manner of sexual reproduction. However, organisms of *Caulerpa* exhibit such a remarkably high degree of morphological variation that many intermediate or transitional growth forms have been recognized (Bφrgesen 1907, 1925; Tandy 1934; Gilbert 1942; Eubank 1946; Taylor 1960; Egerod 1975). The presence of such a great number of intermediate forms makes the boundaries delimiting species of *Caulerpa* uncertain (Gilbert 1942) and causes taxonomic confusion.

Recently, it has been recognized that restriction fragment length polymorphism (RFLP) of chloroplast DNA (cpDNA) is useful to clarify phylogenic relationships of species of land plants (Palmer 1985a) and algae (Palmer 1985a; Olsen 1990). Since cpDNAs of land plants are so conserved in their sequences and gene orders, comparison of their RFLPs is accepted to be an efficient method to reveal their phylogenic relationships at the level of genus and even family (Herrmann *et al.* 1980). In algae, on the other hand, cpDNAs are very divergent compared to land plants. For example, restriction patterns of cpDNAs of *Vaucheria* (Xanthophyceae) show very extensive fragment divergencies at the species level (Kowallik 1989); only one common restriction fragment was detected in five species by digestion with *Eco*RI. In a comparative study of 18 strains of *V. sessilis* Linne von Berg and Kowallik (1988) suggested the possible presence of more than two taxa among them because they thought that the restriction site variations among these strains corresponds to differences found between different genera in higher plants.

In this study we compared restriction patterns of cpDNAs from four species of *Caulerpa* in order to find some clues to resolve the taxonomic confusion of this genus at the interspecific and/or intraspecific levels.

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Materials and Methods

Materials

Caulerpa brachypus and *C. okamurae* were collected at Inamuragasaki in Kanagawa Prefecture (the Pacific Ocean), *C. lentillifera* at Itoman in Okinawa Island, *C. racemosa* var. *clavifera* f. *macrophysa* at Zanpa Cape (the East China Sea) in Okinawa Island and at Ikeijima island (the Pacific Ocean) in the Ryukyu Islands (Fig. 1).

Isolation of chloroplast DNA (cpDNA)

Collected materials were extensively washed in seawater to remove sediment and microscopic epiphytes, and kept in a water tank with aeration until isolation of cpDNAs.

About 200-300 g of materials were used for each isolation. The following procedures were performed at 0°C or 4°C. Materials were cut into small pieces with a knife and transferred into five volumes of an extraction buffer (MS buffer) containing 50 mM Tris-HCl, pH 7.6, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.3 M sucrose, 0.3 M NaCl, 10 mM 2-mercaptoethanol, 1.4 mM phenylmethylsulfonylfluoride (PMSF), 1.2 mM spermidine. This buffer was based on TAN buffer (Nemoto *et al.* 1988), modified to strengthen buffer action and with added NaCl to lower the stickiness of cell lysate according to Grant and Wright (1980). The suspension was filtered through a nylon stocking and pieces of the materials were squeezed in it. The filtrate suspension was filtered through a layer of nylon membrane (100 μm in pore size). The filtrate suspension was centrifuged at 4,500 × g for 15 min. The pellet was resuspended with about 180 m*l* of MS buffer and 20 m*l* of 100% Percoll (Pharmacia) was added to the suspension. After stirring for 30 min, the suspension was centrifuged at 900 × g for 15 min, and the pellet was suspended and centrifuged in the same way. Then the pellet was suspended with MS buffer without PMSF, and final centrifugation was done at 900 × g for 15 min. The pellet was suspended with about 20 m*l* of HTE buffer containing 50 mM Tris-HCl, pH 8.0, 20 mM EDTA, and lysed by addition of 20 mg of Proteinase K (Sigma) and 2% N-Lauroylsalcosine (Sigma), and kept overnight.

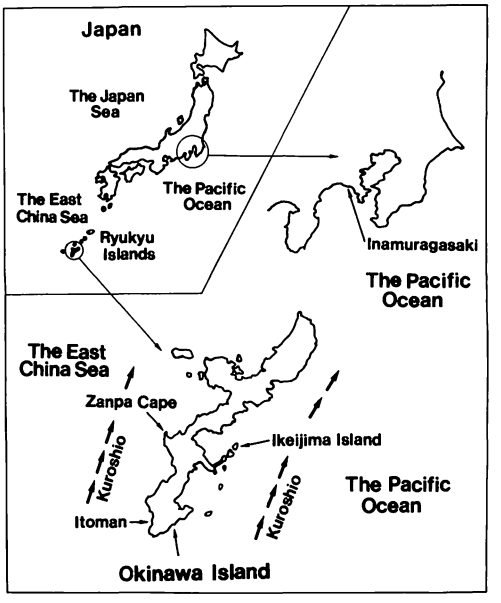

Fig. 1. *Caulerpa* collection sites and the sea water currents (see text).

Nucleic acids were extracted from the lysate by sequential addition of an equal volume of phenol, phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and then extracted with chloroform : isoamyl alcohol (24 : 1). All organic extractions were performed gently for 10 min at room temperature. The aqueous and organic phases were separated by centrifugation at 1,000 rpm for 5 min. The final aqueous phase was dialyzed against TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C. Cesium chloride and Hoechst 33258 dye were added to the solution (1.08 g and 0.3 mg for 1 m*l* of the solution, respectively), and centrifuged at 38,000 rpm for 45 h in a SW41 rotor (Beckman) at 20°C. A band of cpDNA which was formed at a higher (less dense) position than other DNAs in the tube was collected by side-puncture using a 21-gauge needle, extracted five times with isopropanol (equilibrated with a saturated NaCl solution) to remove Hoechst dye and then dialyzed against TE buffer at 4°C. After addition of 2.5 volumes of ethanol it was

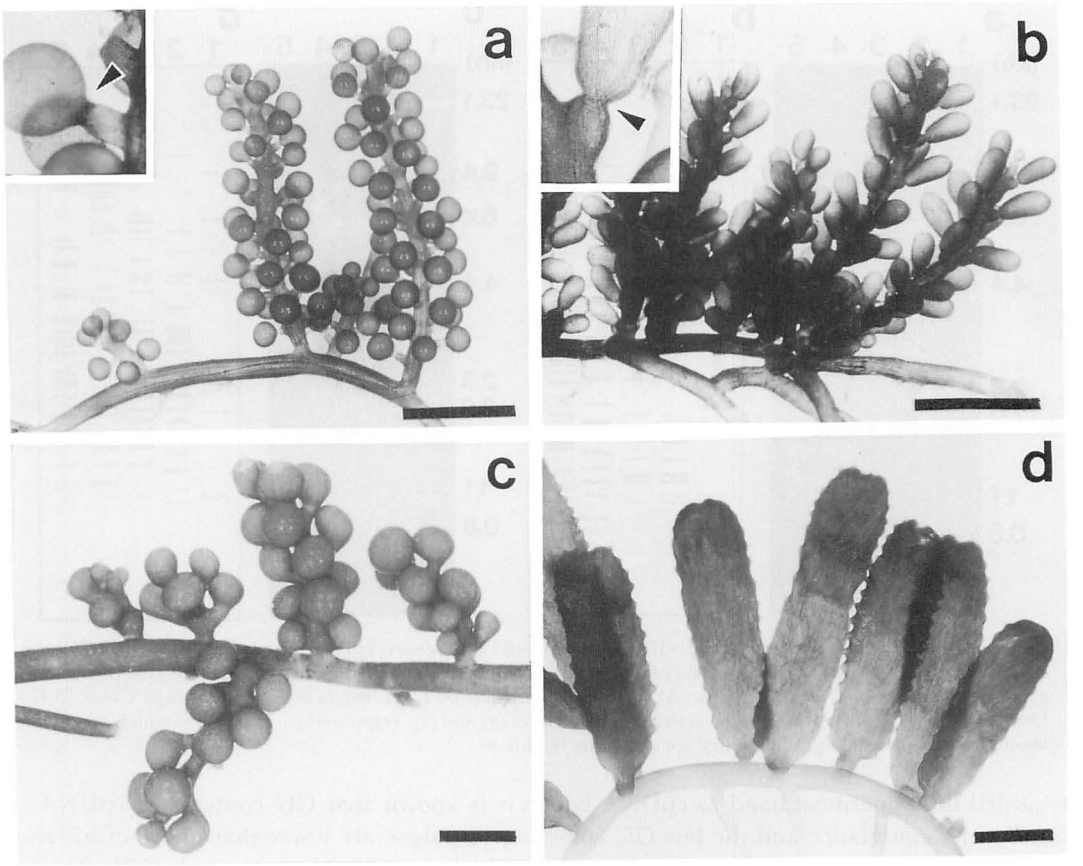

Fig. 2. Photographs of (a) *C. lentillifera*, (b) *C. okamurae*, (c) *C. racemosa* var. *clavifera* f. *macrophysa*, and (d) *C. brachypus*. Insets of a and b show constrictions at the base of ramulus (indicated by arrow heads) of both algae. Bars are 1 cm.

kept at −80°C for 30 min, the cpDNA was collected by centrifugation at 12,000 × g for 15 min. The cpDNA was washed with 70% ethanol and collected by the same centrifugation step, dried, and dissolved in TE buffer, and kept at 4°C.

Restriction endonuclease analysis

Restriction endonucleases were purchased from Takara Shuzo Co. Ltd. and Toyobo Co. Ltd., and used according to the manufacturer's specifications. DNA of λ phage (Takara Shuzo Co. Ltd.) and λ DNA/*Hin*dIII digest-φX174/*Hin*cII digest (Toyobo Co. Ltd.) were used as molecular standards. DNA fragments were resolved at 20 V on 0.9% and/or 1.0% Agarose 1600 (Wako) horizontal gels (13 × 13 cm) in 0.8 × TEA buffer (40 mM Tris-HCl, 32 mM sodium-acetate, 1.6 mM EDTA, pH 8.3) for 14 h. The gels were stained with ethidium bromide and photographs were taken with Minicopy films (Fuji) under UV illumination.

Results and Discussion

Comparison of the restriction patterns of cpDNAs among four Caulerpa species: We obtained chloroplast DNAs (cpDNAs) by centrifugation in the presence of cesium chloride and Hoechst 33258 after extraction of nucleic acids with phenol : chloroform from a crude chloroplast fraction. In the centrifuged tube bands appeared which may correspond to cpDNA, nuclear DNA, mitochondrial DNA (mtDNA) and bacterial DNA (data not shown). We

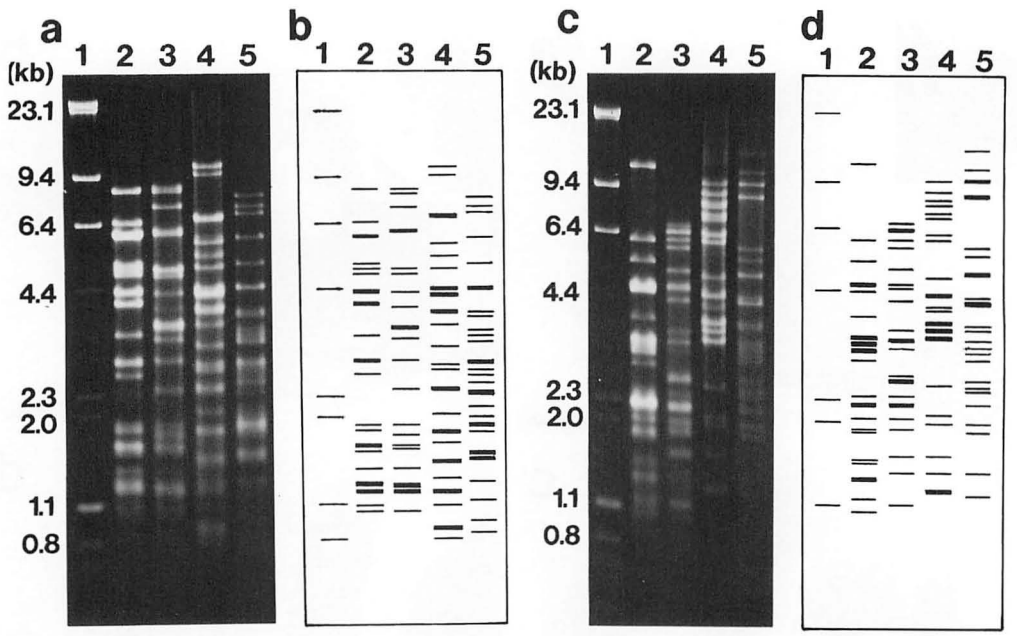

Fig. 3. Restriction patterns of *Hin*dIII (a)- and *Eco*RI (c)-digested cpDNAs from four species of *Caulerpa* separated on 0.9% agarose. Lanes are 1) DNA marker containing *Hin*dIII-digested λ DNA and *Hin*cII-digested ϕX174; 2) *C. lentillifera*; 3) *C. okamurae*; 4) *C. racemosa* var. *clavifera* f. *macrophysa* collected at Zanpa Cape; 5) *C. brachypus*. (b), (d) Illustrations of the restriction patterns of (a) and (c), respectively. Fragments which are much abundant than normal stoichiometory are shown by bold lines.

regarded the uppermost band as cpDNA because of its abundance and the low GC content of cpDNA. Although the contamination of nuclei and mitochondria which were involved with chloroplasts by sticky material (mucilage material) remained in the crude (chloroplast) fraction, the number of nuclei and mitochondria, and the amount of their DNAs was estimated to be much less than those of chloroplasts by examination of the crude fraction with fluorescence microscopy after staining with 4'6-diamidino-2-phenylindole (DAPI) (data not shown). Second, as it is known that GC contents of cpDNA of green algae are lower than those of mtDNA (Rochaix 1978; Hedberg *et al.* 1981; Tymms *et al.* 1985) like land plants (Kolodner and Tewari 1975), cpDNA forms a band in a tube at the higher position than mtDNA after centrifugation in the presence of cesium chloride and Hoechst 33258.

Figure 3 shows the restriction patterns of cpDNAs from four *Caulerpa* species digested with *Hin*dIII and *Eco*RI. The restriction patterns were very heterogeneous among the four species, and there were no bands com-

Table 1. Similarities of cpDNAs of four *Caulerpa*.

*Eco*RI \ *Hin*dIII	*C. lentillifera*	*C. okamurae*	*C. brachypus*	*C. racemosa* var. *clavifera* f. *macrophysa*
C. lentillifera		10	7	4
C. okamurae	6		3	3
C. brachypus	4	7		4
C. racemosa var. *clavifera* f. *macrophysa*	2	4	4	

Numbers of restriction fragments which are identical in sizes are listed when cpDNAs were digested with *Hin*dIII (upper right half of the table) and with *Eco*RI (lower left half of the table).

mon to all the four species. Table 1 shows a list of the number of common fragments among the four species in either digestion with *Eco*RI and *Hin*dIII. When common fragments in both digestions were summed up, *C. lentillifera* shared 16 common fragments with *C. okamurae*, 11 with *C. brachypus*, and 6 with *C. racemosa*. *C. okamurae* shared 16 common fragments with *C. lentillifera*, 10 with *C. brachypus*, and 7 with *C. racemosa*. Thus, *C. lentillifera* and *C. okamurae* shared much more fragments than other two species, and both species shared least common fragments with *C. racemosa*. *C. brachypus* shared 11, 10 and 8 common fragments with *C. lentillifera*, *C. okamurae* and *C. racemosa*, respectively, so that *C. brachypus* had moderate relationships equally with other three species. *C. racemosa* shared most common fragments with *C. brachypus* (8) than with *C. lentillifera* (6) and *C. okamurae* (7). However, *C. brachypus* had less common fragments with *C. racemosa* than with other species. Therefore, *C. racemosa* appears to share least relationships with any of the other three species.

Genome sizes of the cpDNAs which were calculated from restriction patterns also indicated closer relationships between *C. lentillifera* and *C. okamurae* than with the other two species because the genome sizes of the two species were about 90 kb and other two species were more than 100 kb (Table 2).

Besides the characteristics of cpDNA, *C. lentillifera* and *C. okamurae* also have many morphological characteristics in common; (1) they have a constriction at the base of each ramulus (Fig. 2a, b insets), while *C. racemosa* and *C. brachypus* have no such constrictions (Fig. 2c, d); (2) they have chloroplasts with pyrenoid (Hori and Ueda 1967; Calvert *et al.* 1976) in which the cpDNAs are specifically localized (Miyamura and Hori 1991), but the chloroplasts of latter two species lack pyrenoids (Calvert *et al.* 1976); (3) the sizes of their chloroplasts are larger (9–11 μm) than those in the latter (3–5 μm) (Calvert *et al.* 1976). From these features of chloroplast fine structure, Calvert *et al.* (1976) proposed the evolutionary scheme from the microphysa-type chloroplasts bearing pyrenoids (*C. lentillifera* and *C. okamurae*) to the prolifera-type chloroplasts lacking pyrenoids (*C. racemosa* and *C. brachypus*). The evolutionary trend correlates with an increase of genome sizes from the smaller group (*C. lentillifera* and *C. okamurae*) to the larger group (*C. racemosa* and *C. brachypus*).

From these molecular and morphological aspects we concluded *C. lentillifera* and *C. okamurae* are phylogenetically closest among the four species, and *C. racemosa* is furthest from the other three species. Thus, *C. brachypus* appears to be situated in their intermediate position. However, more analyses are required to clarify the precise phylogenic relationships of the four *Caulerpa*. Because of the extreme heterogeneity of restriction patterns, analyses of nucleotide sequencing may be more suitable to this aim.

An extreme diversity of restriction patterns of cpDNAs at the species level is also known in the xanthophyte genus *Vaucheria* (Kowallik 1989). The diversity of caulerpalean cpDNAs is much more extensive than that of another siphonous green algal genus *Bryopsis*, in which only a few restriction fragments differ among three species in the genus (Misonou *et al.* 1989).

The genome sizes of these four caulerpalean cpDNAs were smaller than those of land plants (120–160 kb) (Palmer 1985b). The genome sizes of cpDNAs of *C. lentillifera* and *C. okamurae* were similar to that of *Codium fragile* (89 kb) (Hedberg *et al.* 1981; Manhart

Table 2. Sizes in kb of cpDNAs from four species of *Caulerpa*.

Enzymes	*C. lentillifera*	*C. okamurae*	*C. racemosa* var. *clavifera* f. *macrophysa* collected at Zanpa Cape	*C. brachypus*
*Hin*dIII	91.9	84.1	105.0	92.3
*Eco*RI	90.5	93.3	120.4	128.9

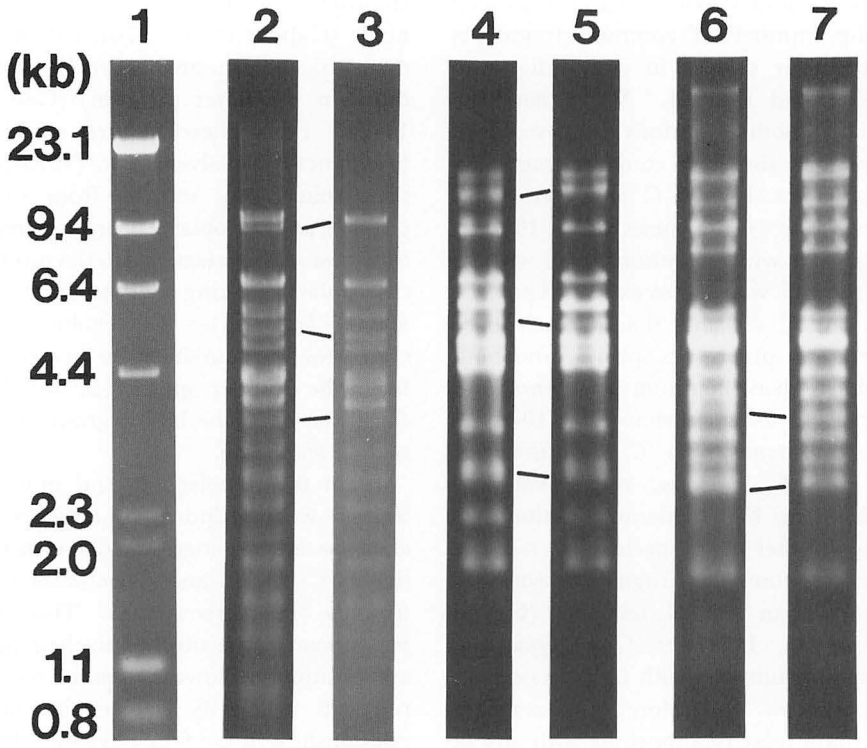

Fig. 4. Restriction patterns of two cpDNAs of *C. racemosa* var. *clavifera* f. *macrophysa* collected at different places. The cpDNAs were isolated from the algae collected at Zanpa Cape (lane 2, 4, 6) and at Ikeijima Island (lane 3, 5, 7), and separated on an 1.0% agarose gel after digestion with *Hin*dIII (lane 2, 3), *Eco*RI (lane 4, 5), and *Eco*RV (lane 6, 7). Lane 1 is the DNA markers containing *Hin*dIII-digested λ DNA and *Hin*cII-digested ϕX174. Fragments which migrated at different positions in the two cpDNAs are indicated by lines.

et al. 1989) which lacks an inverted repeat region on the genome. There is a possibility that cpDNAs of *Caulerpa* also lack an inverted repeat region.

Comparison of restriction patterns of two strains of C. racemosa *var.* clavifera *f.* macrophysa: We compared restriction patterns of two strains of *C. racemosa* var. *clavifera* f. *macrophysa* from different habitats along Okinawa Island (Fig. 1) and examined whether intraspecific variations are found in the cpDNA. Figure 4 shows the restriction patterns of the cpDNAs of the two strains digested by *Hin*dIII, *Eco*RI and *Eco*RV. The two cpDNAs had almost the same fragments, but there are a few fragments which shift in the position of two (*Eco*RV) or three (*Hin*dIII, *Eco*RI) restriction sites (indicated by lines). Since changes of the lengths of these fragments appeared to be canceled in each digestion, the changes may be caused by point mutations, rather than by insertions and/or deletions. The localities where the materials were collected are Zanpa Cape facing the East China Sea and Ikeijima Island facing the Pacific Ocean, respectively (Fig. 1). Judging from geographical separation of the collection sites and the parallel direction of the Kuroshio current along the islands (Watts 1969) genetical interchanges may be rare between both strains, so that the changes of the cpDNA sequence may have been fixed.

In this report, we described that the cpDNAs of four *Caulerpa* species vary remarkably, but the variations existing in a taxon (*C. racemosa* var. *clavifera* f. *macrophysa*) are limited. These results suggest that the analysis of cpDNA variation will be useful in clarifying the systematics and evolutionary relationships

within the genus *Caulerpa*.

The reason for the remarkable divergency of cpDNAs of *Caulerpa* remains to be resolved; whether it is due to differentiation of these species at very early times or rapid molecular evolution of the cpDNAs.

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佐藤征弥*・宮村新一・堀　輝三：嚢状緑藻イワヅタ属 (*Caulerpa*) 葉緑体 DNA の種間および種内変異

沖縄本島で採集したクビレヅタ (*C. lentillifera*), センナリヅタ (*C. racemosa* var. *clavifera* f. *macrophysa*), 神奈川県稲村ヶ崎で採集したフサイワヅタ (*C. okamurae*), ヘライワヅタ (*C. brachypus*) について葉緑体 DNA を単離し，その制限酵素パターンを種間で比較した。これら 4 種の制限酵素パターンは互いに大きく変異していたが，外部形態および葉緑体の構造で多くの共通特徴を持つクビレヅタとフサイワヅタは類似性が高かった。葉緑体 DNA ゲノムサイズは，センナリヅタとヘライワヅタが 100 kb 以上であったのに対し，クビレヅタとフサイワヅタは約 90 kb であった。　また，センナリヅタに関しては沖縄県の残波岬と伊計島で生育地を異にしたものについて制限酵素パターンを比較したところ，僅かながら変異が見られ，種内変異の生じていることが明らかになった。(305 茨城県つくば市天王台1-1-1　筑波大学生物科学系，*現住所：305 茨城県つくば市観音台2-1-2　農業生物資源研究所機能開発部)