

Mem. Natn. Sci. Mus., Tokyo, (7), September 20, 1974

# The *Closterium calosporum* Complex from the Ryukyu Islands

## — Variation and Taxonomical Problems —

By

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市村輝宜\*・渡辺真之\*\*：琉球列島産ミカヅキモの研究

Since WITTROCK (1869) found a small curved species of *Closterium* with spiny zygospores in Sweden and established a new species, *C. calosporum*, similar or slightly deviated forms have been found in other countries, England (WEST & WEST, 1896), France (BOURRELLY & MANGUIN, 1952), Germany (KAISER, 1929; HOMFELD, 1929), South Africa (HODGETTS, 1925), the Soviet Union (SKUJA, 1928; KOSSINSKAJA, 1960), and the United States (TAFT, 1945; COOK, 1963), as well as in Sweden (SKUJA, 1964). In spite of the considerable variation in degree of curvature and shape of the apices of vegetative cells, which are generally believed to be important characters for specific distinction in the genus *Closterium*, all the small curved forms of *Closterium* with spiny zygospores in question are at present included into one species with three varieties, *calosporum*, *maius* (WEST & WEST, 1896), and *brasiliense* (BÖRGESEN, 1890). These varieties are distinguished on the basis of different dimensions of vegetative cell.

The project of Natural History Researches of the Ryukyu Islands organized by the National Science Museum, Tokyo, offered us a favorable opportunity to study the soil samples of the islands (Okinawa and Iriomote-jima), from which we have obtained five sexual strains of algae belonging to the *C. calosporum* complex. The complex is considered to be characterized by small curved vegetative cells and spiny zygospores. The strains have appeared quite variable in vegetative and sexual morphology, as well as in cell size. We have assumed that the strains are suitable materials for the taxonomical study of the complex. Therefore, a comparative study based on modern culture techniques and statistical analyses has been carried out on the strains from Okinawa and Iriomote-jima. Strains from some other areas have been studied for comparison.

### Materials and Methods

All the data reported in this paper were obtained from clonal axenic cultures in the

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synthetic culture media under the defined culture conditions. The following soil samples and designations were used for the sources of the clones from the Ryukyu Islands.

R-5 ; paddy field, Ginama, Okinawa, collected by H. HAGIWARA in June, 1973.

R-9 ; rush field, Yonaha-dake, Okinawa, collected by H. HAGIWARA in June, 1973.

R-11; paddy field, Hoshitate, Iriomote-jima, collected by H. KOYAMA in March, 1973.

R-12; abandoned paddy field, the lower reaches of the Pinai river, Iriomote-jima, collected by H. HAGIWARA in June, 1973.

Clone A-2-22 was isolated in axenic culture from population samples obtained at a small pond, Tahara, Tsukude-mura, Aichi-ken in October, 1972. Clone Akan-46 (WATANABE, 1974) was isolated in axenic culture from population samples collected at a bog water near Lake Akan in September, 1973. Clones C-318 (COOK 1193, IU 1146), C-319 (COOK 1204, IU 1086), and C-320 (COOK 1440, IU 1087) were obtained from the Culture Collection of Algae, Institute of Applied Microbiology, University of Tokyo, and these clones were originally isolated in uni-algal culture from the soil samples collected in the United States by COOK (1963). COOK's clones had been maintained in PRINGSHEIM's soil-water medium at the Culture Collection of Algae, Department of Botany, Indiana University, Bloomington, Indiana, until Prof. R. C. STARR kindly transferred the cultures to one of us in 1969 and they were purified into axenic cultures in the synthetic culture media for the present study

Table 1. Synthetic culture media for *Closterium*.

Salt	VT <sup>(1)</sup>	CA	MI
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	11.78 mg	2.00 mg	—
KNO <sub>3</sub>	—	10.00	—
NH <sub>4</sub> NO <sub>3</sub>	—	5.00	—
β-Na <sub>2</sub> glycerophosphate·5H <sub>2</sub> O	5.00	3.00	5.00 mg
MgSO <sub>4</sub> ·7H <sub>2</sub> O	4.00	2.00	4.00
KCl	5.00	—	—
CaCl <sub>2</sub> ·2H <sub>2</sub> O	—	—	10.00
Vitamin B <sub>12</sub>	0.01 μg	0.01 μg	0.01 μg
Biotin	0.01	0.01	0.01
Thiamine HCl	1.00	1.00	1.00
P IV metals <sup>(1)</sup>	0.30 ml	0.10 ml	0.30 ml
Fe (as EDTA; 1:1 molar) <sup>(2)</sup>	—	0.10 mg	—
Glycylglycine	50.00 mg	—	—
HEPES <sup>(3)</sup>	—	40.00	40.00 mg
Distilled H <sub>2</sub> O	99.70 ml	99.90 ml	99.70 ml
pH adjusted with NaOH	7.5	7.2	6.8–8.0

(1) PROVASOLI & PINTNER's volvox medium (1959); 1 ml of P IV metals contains Na<sub>2</sub> EDTA, 1 mg; Fe (as Cl<sup>-</sup>), 0.04 mg; Mn (as Cl<sup>-</sup>), 0.01 mg; Zn (as Cl<sup>-</sup>), 0.01 mg; Co (as Cl<sup>-</sup>), 0.001 mg; Mo (as Cl<sup>-</sup>), 0.005 mg.

(2) Dissolve 351 mg of Fe (NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O and 330 mg Na<sub>2</sub>EDTA in 500 ml H<sub>2</sub>O; 1 ml of this solution=0.1 mg Fe (PROVASOLI, 1966).

(3) This salt can be replaced by MES for lower pH (5.6–6.8).

in February, 1974.

The pipette-washing method was employed for isolation of clonal axenic cultures from Petri dishes, in which a small portion of air-dried soil was rewetted with distilled water and it was kept for approximately two weeks under the culture conditions. For statistical analyses of quantitative variation in vegetative morphology, cells were allowed to grow in test tubes containing 10 ml of CA for three weeks and they were fixed 8 hours after the beginning of light period by three drops per tube of formalin solution. Concerning width and distance between apices, fifty cells per clone were measured under the microscope with screw micrometer, and as to curvature, twenty cells per clone were measured by the method of NISHIHAMA & ICHIMURA (1974). The replacement method of ICHIMURA (1971) was employed for induction of sexual reproduction, but the washing procedure was omitted. The culture media used are shown in Table 1. All the culture experiments were carried out at 23–25°C. The cultures were illuminated by a bank of cool-white fluorescent lamps on a cycle of 16 hours light and 8 hours dark. Light intensity was selected at about 4,000 lux for vegetative growth and at about 10,000 lux for sexual reproduction.

### Strains from the Ryukyu Islands

**Heterothallic strains from R-5 and R-11** A total of 25 clones of small curved *Closterium* (Pl. 13-A) were obtained from R-5 and 10 clones from R-11. All the clones grew well in CA, but scarcely in VT. No sign of sexual reproduction was observed in clonal culture in any medium tested (including MI). In strain R-5, heterothallism was easily recognized, since many zygospores were always observed within 5 days when two clones of opposite mating type in any combination were mixed in MI. It was uncovered that 9 clones were *plus* and the rest *minus*. In strain R-11, however, we noticed that sexual reaction did not occur even when two clones of opposite mating type, in certain pairs, were mixed under the same conditions. The results of several trial crossings among the clones of R-11 and test crossings of them with *plus* and *minus* clones of R-5 and of R-11 are shown in Table 2. In the cases when zygospores were observed, zygospores formed in pairs of R-11 were considerably smaller in number than in pairs of R-5. Especially a very few zygospores were formed in the pair of R-11-9 and R-11-11. On the other hand, a rather large number of zygospores were formed in the pair of R-11-5 and R-11-6. Then the pair was tested at various pH of MI. A considerable number of zygospores were formed in the range from 6.8 to 8.0, but the number decreased at lower pH (5.5–6.5). Concerning the effect of pH on the sexual reproduction, the same sort of results were obtained in the pair of R-5-2 and R-5-3. The results of intercrossing between *plus* and *minus* clones of R-5 and R-11 are shown in Table 3. This table shows only the presence or absence of zygospores in test tubes when observed by a stereomicroscope 10 days after mixing two clones in MI at pH 8.0. It should be noted that a very few zygospores were present in crosses between R-11-9 and R-11-6, R-11-10 and R-5-24, R-11-10 and R-5-26, and R-11-10 and R-11-6. It is suggested that the *plus* clones of R-11 have only weak sexual potential, although the cause and the meanings have not yet been thoroughly

Table 2. Trial cross in R-11 clones and test cross of R-11 clones with *plus* and *minus* clones of R-5 and R-11.

No. of experiments	MI at pH	Test clone	Clone number of R-11										
			5	6	7	8	9	10	11	12	13	14	
Trial 1	8.0	R-11-5	—	Z	Z	—	—	—	—	—	—	—	—
2	7.6	R-11-7	Z	—	—	—	0	0	—	—	—	—	—
3	7.5	R-11-9	—	—	—	—	—	—	Z	—	—	—	—
4	8.0	R-11-14	—	—	—	—	0	0	—	—	—	—	—
Test 1	8.0	R-5-2	Z	—	—	—	0	0	—	—	—	—	—
		R-5-3	—	Z	Z	Z	—	—	Z	Z	Z	Z	Z
		R-5-2	Z	—	—	—	0	Z	—	—	—	—	—
		R-5-3	—	Z	Z	Z	—	—	Z	Z	Z	Z	Z
		R-11-6	Z	—	—	—	0	0	—	—	—	—	—
		R-11-5	—	Z	Z	0	—	—	Z	Z	Z	Z	

Z, zygospores were observed; 0, zygospore was not observed when two clones of opposite mating type were mixed; —, zygospore was not observed when two clones of the same mating type were mixed.

Table 3. Intercross between *plus* and *minus* clones of R-5 and R-11.

<i>Minus</i> clone	<i>Plus</i> clone					
	R-5-3	R-5-5	R-5-6	R-11-5	R-11-9	R-11-10
R-5-2	Z	Z	Z	Z	0	Z
R-5-24	Z	Z	Z	Z	0	Z
R-5-26	Z	Z	Z	Z	Z	Z
R-11-6	Z	Z	Z	Z	Z	Z
R-11-7	Z	Z	Z	Z	Z	0
R-11-11	Z	Z	Z	Z	0	Z

Z, zygospores were observed; 0, zygospore was not observed. MI, pH 8.0.

investigated. Nevertheless, the results of intercrossing between the two strains seem to indicate that the two local populations of the small curved species of *Closterium* in Ginama and Hoshitate are not reproductively isolated, although the two islands, Okinawa and Iriomote-jima, are separated by more than 400 km.

The vegetative cells of the two strains have been quite similar in qualitative morphological characters. Considerable constancy within a clone and also among the clones has been observed in the following characters; the shape of the apices, which are rounded-truncate, and the presence of a small dot like wall thickening at the apex. The quantitative morphological characters have been statistically analyzed on 10 representative clones from strain R-5 and 5 representative clones from strain R-11 (Table 4.). In these clonal cultures, one standard deviation (SD) of width was in the range from 0.3 to 0.7  $\mu$ , and SD of distance was from 3.8 to 8.4  $\mu$ . Coefficient of variability (CV) of width was in the range from 2.7 to 5.7%, and CV of distance was from 4.4 to 9.0%. Thus it can be said that

Table 4. Variation in vegetative characters among heterothallic clones from R-5 and R-11.

Clone	Sex	Width ( $\mu$ )					Distance between apices ( $\mu$ )					Curvature ( $^{\circ}$ )			Distance /width
		Min	Mean	Max	SD	CV (%)	Min	Mean	Max	SD	CV (%)	Min	Mean	Max	
R-5-3	+	12.0	12.7	13.5	0.3	2.7	82.0	93.6	110.0	5.9	6.4	100	116	128	6.5-8.5
R-5-5	+	12.3	13.0	14.0	0.3	2.5	76.8	90.5	102.3	5.7	6.3	108	116	128	6.3-7.5
R-5-6	+	11.5	13.0	13.8	0.4	3.4	79.8	93.8	117.5	7.1	7.6	108	116	124	6.5-7.8
R-5-25	+	11.3	12.0	13.3	0.5	4.1	78.0	87.6	96.8	3.8	4.4	108	120	132	6.3-7.9
R-5-28	+	12.0	12.9	15.3	0.7	5.4	82.5	92.3	102.8	4.7	5.1	100	120	132	6.4-8.5
R-5-2	-	11.5	12.8	14.8	0.7	5.7	75.3	93.3	120.0	8.4	9.0	108	116	124	5.8-8.9
R-5-24	-	11.3	12.6	13.3	0.5	3.7	79.5	92.1	102.5	5.3	5.8	92	116	136	6.5-8.2
R-5-26	-	12.3	13.7	14.5	0.5	3.4	72.5	84.7	100.5	5.4	6.3	104	116	132	5.4-7.6
R-5-27	-	11.5	12.6	14.3	0.5	4.2	73.8	88.4	104.8	7.0	7.9	104	120	136	6.0-7.8
R-5-30	-	11.8	12.5	14.0	0.5	3.8	80.0	98.7	113.5	6.6	6.7	100	108	116	7.0-9.6
R-11-5	+	11.8	12.7	14.0	0.5	4.1	72.3	84.0	95.8	5.6	6.6	112	116	124	5.7-7.3
R-11-9	+	13.0	14.0	15.0	0.5	3.4	80.3	91.7	103.5	5.3	5.7	104	116	124	6.0-7.3
R-11-10	+	13.0	14.0	15.0	0.5	3.3	74.0	88.7	103.0	6.4	7.3	108	116	128	5.4-7.2
R-11-6	-	11.5	13.3	14.5	0.7	4.9	82.0	92.6	105.3	5.3	5.7	104	112	128	6.4-7.8
R-11-7	-	12.5	13.6	14.5	0.4	2.7	84.8	94.4	104.8	4.8	5.0	92	112	120	6.3-7.5

distance is more variable than width. The largest mean value in distance and the smallest value in curvature were obtained in R-5-30. There seems to be some correlation between distance and curvature. In other respects, we have not been able to recognize any significant difference in statistical values of the characters measured.

The sexual morphology (Pl. 14-A) of strains R-5 and R-11 has not shown any difference. The characteristic spiny zygospore is formed within a thin tough membrane between the two conjugating cells. The two semicell walls of a empty gametangium are partially separated, but they are always joined by a hinge.

**Homothallic strains from R-9** A total of 27 clones were obtained from R-9. Vegetative cells of 20 clones were slender (Pl. 13-D) than those of the other 7 clones (Pl. 13-E). Of these 20 clones with slender cells, four clones were originally isolated with VT, in which they grew very poorly, but formed zygospores in clonal culture about one month after the inoculation of single cells. The other 16 clones were isolated with CA, in which they grew vegetatively to a certain extent, but scarcely formed zygospores. The clones cultured in CA were tested to induce sexual reproduction in MI at pH 8.0. Most clones did not show any sign of sexual reproduction, but only a few clones formed a very small number of zygospores.

The clones with slender cells can be successfully induced to form many zygospores in MI at pH 6.5. The clones isolated with VT and those with CA are quite similar in sexual morphology (Pl. 14-C). The characteristic spiny zygospore is formed within a comparatively large vesicle between the two conjugating cells. In some cases, another thin membranous structure can be seen between the outer thin membrane and the zygospore (Pl. 14-C). In most cases, the two semicell walls of a empty gametangium are separated by a

Table 5. Variation in vegetative characters among homothallic clones from R-9.

Clone	Width ( $\mu$ )					Distance between apices ( $\mu$ )					Curvature ( $^{\circ}$ )			D/W
	Min	Mean	Max	SD	CV (%)	Min	Mean	Max	SD	CV (%)	Min	Mean	Max	
Slender group														
R-9-5	16.5	17.3	18.3	0.4	2.1	127.5	148.9	168.3	8.5	5.7	84	96	104	7.5- 9.4
R-9-6	17.0	17.7	18.8	0.3	1.9	127.5	164.2	181.8	10.4	6.4	84	96	116	7.3-10.0
R-9-12	16.0	17.4	17.8	0.4	2.4	145.0	154.8	172.8	7.8	5.0	84	96	112	7.9- 9.4
R-9-13	14.5	16.0	17.0	0.7	4.2	117.8	141.6	169.5	11.2	7.9	88	100	116	7.8-11.1
R-9-22	16.8	17.1	17.5	0.2	1.4	125.0	145.2	165.5	10.4	7.1	84	96	112	7.4- 9.4
Broader group														
R-9-10	24.8	26.0	27.5	0.5	1.9	142.3	178.6	195.3	10.0	5.6	124	132	140	6.2- 7.5
R-9-11	23.8	25.3	26.8	0.6	2.4	148.3	168.5	193.5	9.4	5.6	120	136	148	6.1- 7.5
R-9-40	24.5	26.3	28.5	1.1	4.1	160.0	179.9	201.5	10.2	5.6	120	132	140	6.3- 8.1
R-9-42	24.0	25.3	26.5	0.5	1.9	156.3	177.6	201.8	9.3	5.2	120	132	144	6.6- 8.0

ring of newly formed thin wall, on which a gamete exit pore is opened. Constancy within a clone and also among the clones with the same sexual morphology has been observed in the following vegetative characters; the shape of the apices, which are clearly truncated, and the presence of a dot like wall thickening at the apex. The quantitative characters of the vegetative cells have been statistically investigated on 5 representative clones (Table 5.). The statistical values have shown that R-9-13 is deviated to considerable extent from the other 4 clones.

The seven clones with broader cells grew to the limited extent in CA, but scarcely in VT. Occasionally a few cells showed the sign of sexual reproduction in clonal culture when the culture aged, especially in VT; a few cells conjugated, but they failed to form zygospores. Induction of the sexual reproduction with MI at pH 7.5 was unsuccessful. Many zygospores were observed in MI at pH 6.5 approximately one month after the replacement. The sexual morphology of the strain with broader cells is shown in Pl. 14-F. The characteristic spiny zygospore is formed within a thin vesicle between the two conjugating cells. The two semicell walls of a empty gametangium are never completely separated. The exit pore of gametic protoplast can be seen on the newly formed thin wall between the two semicell walls. A dot like wall thickening can be seen at the apex of every vegetative cell of all the clones with broader cells. The apices appear to be slightly truncated in most of the cells, but they may also appear to be acutely rounded in some cells (Pl. 13-E). The quantitative characters of the vegetative cells have been statistically investigated on 4 representative clones (Table 5.).

**Homothallic strain from R-12** A total of 5 clones were obtained from R-12. In CA, all the isolates did not grow well vegetatively, but they reproduced sexually to form zygospores about one month after the inoculation of single cells. Two months after the isolation, however, only clone R-12-2 grew vegetatively to a considerable extent in CA. The vegetative cells (Pl. 13-H) of the clone have been observed to be fairly constant in the following characters; the shape of the apices which appears to be truncated, and the presence

of a dot like spot at the apex. Occasionally, giant cells (Pl. 13-G) have been observed in the clone. These giant cells were largely deviated in size from the normal cells, but the shape of the apices of them did not show much difference from that of the normal cells. Measurements in width and distance of the giant cells are shown in the following 4 examples;  $12.8 \times 80.0$ ,  $13.0 \times 112.5$ ,  $13.5 \times 80.0$ , and  $14.3 \times 87.5$  in  $\mu$ . The size of them seems to be very variable. The statistical analysis of the quantitative characters of the vegetative cells of the clone, which will be described in the following section, has been done on 50 normal cells. In all the clones from R-12, the zygospores have been observed to have poorly developed mammillae, but not true spines, on their walls in CA, although most of the zygospores were matured. Development of mammillae was improved in CA at lower pH (5.6-6.8). The shape of the "spine", however, was still rounded protuberances under the best conditions (Pl. 14-I). It has been observed that the two semicell walls of a gametangium are slightly separated by a ring of newly formed thin wall, and that the zygospore is surrounded by a thin vesicle between the two conjugating cells.

### Comparative Studies of Morphology of Strains from Different Localities

A total of 10 strains from different localities have been studied to compare the vegetative and sexual morphology. Statistical analyses have been done on the quantitative characters of the vegetative cells (Table 6.). In order to compare the characters of a local population with those of other local populations, one hundred cells were randomly chosen from each of the strains, in which more than two clones had been statistically investigated in the foregoing section. The strains are arranged in the order of mean values of the width from the top of the table. In the 10 strains studied, SD of width was in the range from 0.3 to  $0.8 \mu$ , and there seemed to be no correlation between the larger value of mean and the larger value of SD. SD of distance was in the range from 3.4 to  $14.5 \mu$ , and SD

Table 6. Variation in vegetative characters among strains from different localities.

Strain	Width ( $\mu$ )					Distance between apices ( $\mu$ )					Curvature ( $^{\circ}$ )			No. of pyrenoides per plastid*	D/W
	Min	Mean	Max	SD	CV (%)	Min	Mean	Max	SD	CV (%)	Min	Mean	Max		
R-12-2	9.3	10.4	11.5	0.6	5.3	47.8	57.1	65.8	3.4	5.9	108	120	140	1-2	4.7- 6.1
C-318	10.0	10.5	11.5	0.3	3.0	72.3	81.5	91.3	4.6	5.6	136	148	164	2-4	6.9- 8.5
A-2-22	10.8	11.5	12.5	0.5	4.1	65.5	75.7	89.8	5.8	7.7	92	104	112	1-2	6.0- 7.8
R-5	11.3	12.7	15.3	0.6	4.9	72.5	91.5	120.0	6.5	7.1	92	116	136	1-2(3)	5.4- 9.6
R-11	11.5	13.5	15.0	0.8	5.6	72.3	90.0	105.3	7.0	7.7	92	114	128	(1)2-3	5.4- 7.8
C-320	13.3	14.0	14.8	0.3	2.2	113.8	128.1	139.8	6.3	4.9	68	80	96	2-3(4)	8.3-10.1
Akan 46	14.3	15.2	16.8	0.6	3.9	116.3	150.0	188.5	14.5	8.5	76	96	112	2-5(6)	9.0-13.0
C-319	15.3	16.0	16.5	0.3	1.8	112.0	121.3	137.5	5.2	4.3	96	108	124	(1)2-3	6.9- 8.2
R-9 slender	14.5	17.1	18.8	0.8	4.5	117.8	150.4	181.8	12.7	8.4	84	96	116	(1)2-5(6)	7.3-11.1
R-9 broader	23.8	25.6	28.5	0.6	2.3	142.0	175.0	201.8	13.0	7.4	120	133	148	(2)3-6(5)	6.1- 8.0

\* Based on 10 or more cells.

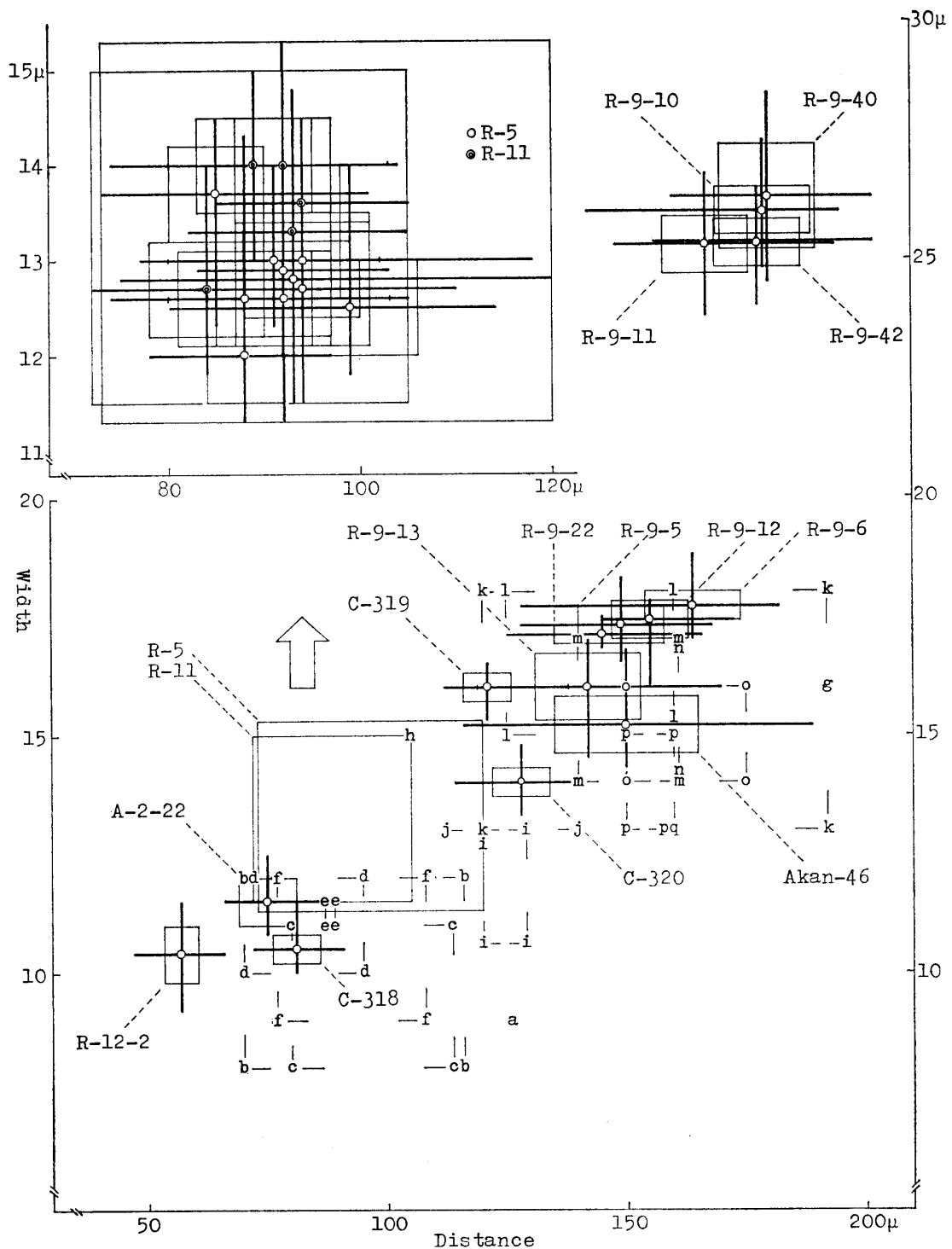


Fig. 1. Graphic analysis of variations in width and distance between apices of vegetative cells. *C. calosporum*: b (KRIEGER, 1935), c (SKUJA, 1928), d (WITTORCK, 1869), f (WEST & WEST, 1904), g, h (HOMFELD, 1929); *C. calosporum* var. *brasiliense*: a (HOMFELD, 1929); *C. calosporum* var. *galiciense*: j (KAISER, 1929); *C. calosporum* var. *maius*: k (KRIEGER, 1935), l (BOURRELLY & MANGUIN, 1952), p (SKUJA, 1964), q (TAFT, 1945); *C. calosporum* f. *major*: m (WEST & WEST, 1904), n (WEST & WEST, 1896), o (SKUJA, 1928); *C. spinosporum*: i (HODGETTS, 1925); *C. spinosporum* var. *minus*: e (HODGETTS, 1925); crossed square, see the text.

was considerably large among the strains which have mean values over  $150\mu$ , such as Akan-46 and the two strains of R-9. CV of width was in the range from 1.8 to 5.6% and CV of distance was from 4.3 to 8.4%. It seems that width is less variable than distance. From mean values of curvature, it can be said that the vegetative cells of C-318 are the most curved and those of C-320 are the least.

Variations in width and distance among the 10 strains, and also among the clones within a given strain, can be visually compared in Fig. 1. When an attention is given to crossed squares three groups roughly defined can be recognized. The top group is composed of the four clones of strain R-9 with broader cells. The strain is markedly separated from any other strain. The middle group includes 4 strains; R-9 with slender cells, C-319, Akan-46, and C-320. Within this group, strain R-9 with slender cells is the largest in width and also in distance. C-320 is the smallest in width, and C-319 is the smallest in length. When an attention is given to squares, by which SDs of width and distance are shown, C-319 and C-320 are completely separated from any other strain. There is also a gap between the group of the four clones of R-9 and the pair of R-9-13 and Akan-46. R-9-13 is the most deviated clone within strain R-9 with slender cells. However, ranges in width and distance, which are shown by crosses, overlap each other in these 4 strains. The bottom group includes 5 strains; R-11, R-5, A-2-22, C-318, and R-12-2. Within this group, strain R-11 is the largest in width, and strain R-5 in distance. R-12-2 is the smallest in width and also in distance. The squares of R-12-2 and C-318 are clearly separated from others.

Quantitative variation in morphological characters of the sexual cells has not been able to be studied under the same constant conditions in all the strains, because of different pH optima for their sexual reproduction. The measurements of size of zygospores have shown that there was some correlation between size of zygospore and that of vegetative cell (Table 7.). Any significant difference has not been recognized between the two strains of R-5 and R-11 in their sexual morphology, too.

Table 7. Variation in sexual characters among strains from different localities.

Strain	Condition	Zygospore without spines										Length of spine ( $\mu$ )			
		Major axis ( $\mu$ )					Minor axis ( $\mu$ )					Min		Max	
		Min	Mean	Max	SD (%)	Min	Mean	Max	SD (%)	Min	Mean	Max	SD (%)		
R-12-2	CA	6.8	21.3	22.7	25.3	1.1	20.0	21.8	24.8	1.5	1.3	2.0	3.0	0.4	
A-2-22	MI	7.0	24.8	26.9	28.0	1.0	23.0	25.2	27.0	1.3	1.3	2.2	3.3	0.6	
R-11-5 ×R-11-6	MI	8.0	25.0	27.2	28.8	1.3	23.0	24.7	27.3	1.5	1.5	2.3	3.3	0.4	
R-11-5 ×R-5-27	MI	8.0	25.0	27.7	32.0	2.0	23.8	25.9	27.5	1.4	2.3	3.1	3.8	0.4	
R-5-2 ×R-5-3	MI	8.0	24.3	27.9	30.0	1.8	23.8	25.9	28.8	1.7	1.8	3.2	5.0	0.7	
Akan 46	CA	7.2	31.5	33.2	35.0	1.1	28.8	30.8	32.5	1.3	2.3	3.1	4.0	0.5	
R-9-6	MI	6.5	32.5	36.1	41.5	3.4	29.3	33.9	37.5	2.5	2.5	4.0	5.0	0.9	
R-9-11	MI	6.5	42.5	44.5	47.5	1.7	37.5	41.3	45.0	2.5	2.5	4.2	6.8	1.2	

In each strain, ten mature zygospores, which had been incubated for more than 10 days at the indicated condition, were measured. As to the length of spine, two longer spines were selected from each of the ten zygospores.

### Discussion

The desmid flora of the Ryukyu Islands has been studied by some Japanese phycologists (OKADA, 1943; HIRANO, 1963; YAMAGISHI, 1969; NAKANO, 1970). Most previous reports, however, are based on the fixed materials collected from the fields. Such materials rarely contain zygospores of desmids. Actually, the occurrence of organisms belonging to the *C. calosporum* complex in the Ryukyu Islands seems to be confirmed for the first time by the present work. The characteristic spines on the zygospores observed made it possible to recognize them with certainty.

The small curved forms of *Closterium* with spiny zygospores from the islands present many interesting biological problems including the taxonomical problems in the genus *Closterium*.

The common characteristics of them is only that the spiny zygospore is surrounded by a thin hyaline vesicle formed between the two conjugating cells. The presence of the vesicle has been observed also in the strains from other geographical areas. In his original description of *C. calosporum*, WITTROCK (1869) has described that the conjugation vesicle of the new species gradually disappears by the time of zygospore maturation, in contrast with the vesicle of *C. parvulum* which is comparatively tough and remains throughout the zygospore maturation. Under the axenic culture conditions, the vesicle is constantly present in all the strains, around the matured zygospores, although there seems to be some variations in toughness of the membranes.

From the available illustrations, we have assumed that there are considerable variations in shape and also in length of the spines of zygospores among the small curved forms which have been reported as *C. calosporum* and its varieties, and that the shape of the spine might be a useful criterion for distinction of taxa. Actually it has been observed that there is a marked difference in the spines between the heterothallic strains (Pl. 14-A) and the homothallic strains (Pl. 14-I). However, it has also been observed that development of the spines is strongly influenced by the environmental factors such as pH of the medium. In A-2-22, the zygospores formed in MI at pH 7.0 had poorly developed mammillae on the wall or had smooth walls (Pl. 14-H). Development of the protuberances on the zygospore walls was largely improved to form true spines in MI at pH 5.6 (Pl. 14-G). In most of the strains studied, occasionally formed parthenospores bore the same characteristic spines as those of the normal zygospores at the optimal pH range (Pl. 14-B & D). Before the usefulness of the spines as a taxonomic criterion can be properly evaluated, it is necessary, first of all, to determine optimal conditions for sexual reproduction of each strain.

The available informations on the organisms, which can be positively identified as the *C. calosporum* complex from the descriptions with illustration of the characteristic spiny zygospores, show that dimensions of the vegetative cells considerably vary among the local populations (Fig. 1). Same small letters in this figure indicate ranges in width and distance which have been described by a given author. The dimensions of *C. calosporum* which have been described by WITTROCK (1869) are indicated by the four letters

of *d*. The ranges of C-318 are entirely surrounded by the four letters. This means that the vegetative cells of the clone agree well in dimensions with the original description. In addition, judging from his illustration, the vegetative cells of the clone agree fairly well with WITTRÖCK's material in curvature and also in the shape of acutely rounded apices. However, the "spines" on the zygosporangia of C-318 are merely poorly-developed mammillae, in contrast with the well-developed spines on the zygosporangia of WITTRÖCK's material. We have observed the sexual morphology of C-318 (Pl. 14-E) in uni-algal cultures using PRINGSHEIM's soil-water medium so far. The vegetative cells of A-2-22 agree considerably well in dimensions with the original description of *C. calosporum*, but, in curvature, they greatly differ from it. The dimensions of *C. calosporum* which have been delimited by WEST & WEST (1904) and by KRIEGER (1935) are indicated in Fig. 1 by the four letters of *f* and *b*, respectively. The vegetative cells of R-12-2 agree well in width, but not in distance, with the diagnoses of the species in the two major monographs.

The apices of the vegetative cells appear to be truncated in A-2-22 and also in R-12-2. By this nature of the apices, the two strains may be considered to belong to different taxa from *C. calosporum*. HODGETTS (1925) has described two taxa of *Closterium* with spiny zygosporangia, *C. spinosporum* and its variety *minus*, of which vegetative cells closely resemble a small form of *C. diana* and the apices are obliquely truncated. According to him, HODGETTS' materials appear to resemble closely *C. paradoxum*, but to differ from it only in the nature of zygosporangial spines. We have not been able to consult with WILLE's (1880) description of *C. paradoxum* at this time. As to the taxonomic value of the nature of the spines, however, we have the opinion, as discussed above, that the evaluation should be awaited for the time when we will have enough knowledge of the environmental modification or phenotypical plasticity of the shape of the spines. Therefore, we can not decide at this moment whether *C. paradoxum* and *C. spinosporum* are conspecific or not. In any way, the vegetative cells of A-2-22 agree well in width and almost in distance with those of *C. spinosporum* var. *minus*, but the former appears to be slightly shorter and less curved than the latter.

Dimensions of the vegetative cells of the middle group of the strains are almost all included in the space indicated by the four letters of *k* in Fig. 1, which shows the ranges of the dimensions of *C. calosporum* var. *maius* described by KRIEGER (1935). Although considerable variations in the dimensions can be seen, the apex of the vegetative cell is more or less clearly truncated and has a dot like wall thickening in all the strains, and the descriptions of the variety by KRIEGER (1935) and by several other authors (SKUJA, 1928, 1964; HOMFELD, 1929; KAISER, 1929; TAFT, 1945; BOURRELLY & MANGUIN, 1952) agree well at this respect. WEST & WEST (1896, 1904), however, have not mentioned on the nature of apices of vegetative cells in their descriptions of *C. calosporum* f. *major*.

The two heterothallic strains of R-5 and R-11 can be regarded to belong to the same single species, since members of the two strains are potentially linked with each other through the sexual reproduction. The vegetative cells of the two strains are intermediate between those of *C. calosporum* and its variety *maius*. We have not found any known taxon to which we can properly identify the species, although it is supposed to be the organism

more or less closely related to *C. calosporum*.

From dimensions and curvature of the vegetative cells, we may be able to identify the broader cells of R-9 as *C. diana*e or *C. parvulum* var. *maius*. However, the spiny zygospores such as seen in the strain have never been observed in both of the two species. Therefore, the strain is considered to belong to the species which has never been described so far. The diagnosis of the new species will be published elsewhere.

### Acknowledgments

We wish to express our sincere thanks to Dr. Tuguo TATEOKA for critically reading the manuscript and to Miss Sachiko NIIBORI and Mrs. Kyoko SATO for their invaluable technical assistance in preparing the present paper.

We also wish to express our sincere thanks to Dr. Hiroshige KOYAMA, Dr. Hiromitsu HAGIWARA, and Dr. Toshiyuki NAKAIKE, National Science Museum, for the soil samples from the Ryukyu Islands; to Dr. R. C. STARR, Indiana University, for the three strains studied by Dr. P. W. COOK; to Dr. J. A. WEST, University of California, for his suggestion on GOOD's buffers for our synthetic media; to Dr. Minoru HIRANO, Kyoto University and also to Dr. Takaaki YAMAGISHI, Nihon University, for the literature on the taxonomical problems; and to Mr. Chisaku ASAI and Mr. Shigehisa MIURA for their help during our field trip in Aichi-ken, 1972.

### 要 約

細胞が小型で接合子にトゲのあるミカヅキモ *Closterium calosporum* complex はこれまでに世界各地から報告されている。KRIEGER (1935) はそれらを *C. calosporum* とその変種 *maius* と *brasiliense* とに統合整理した。その後多くの人達はその便利さのためか彼の分類系に従って来た。われわれは琉球列島のチリモ類のフロラを調査する過程で、上記の分類群に属する藻と、いくつかの特徴でそれらと異なる藻の多数のクローンを無菌的に分離することができた。これらの琉球産クローンと合衆国産、北海道産、愛知県産の無菌クローンとについて、一定条件下で培養し統計的方法を用いて栄養形態および接合形態の比較をし、*C. calosporum* complex の解析を試みた。本研究は多くの異質の生物群を機械的に統合してしまったと思われる KRIEGER (1935) の分類系の再検討の一環として行なわれた。

材料は沖縄本島の宜名真と与那覇岳山麓および西表島の干立とピナイ川下流の4ヶ所の水田土壌と北海道阿寒町、愛知県作手村および合衆国の土壌から分離された。これらのクローンは形態、産地などにより10系統に区別された。なお、合衆国産の系統は P. W. COOK によって分離され、PRINGSHEIM の二相培地を用いて10年以上継代培養されたものである。本研究ではそれらを合成培地で無菌的に培養した。COOK (1963) の測定値と本報告の測定値とを比較すると、ほとんど差違が認められない。したがって本報告で問題とする諸形質は継代培養において安定したものであると考えてよいと思われる。

培養には合成培地 (Table 1) を用いた。温度は 23-25°C、光源には白色蛍光灯を用い、明暗の周期(16:8)の下で、栄養増殖には約 4000 lux、接合誘起には約 10000 lux の照度で培養した。

栄養形態の測定は3週間栄養増殖をさせた細胞を明期に入って約8時間後にフォルマリンにより固定して行なった。この状態では分裂直後の細胞は見られず、ほとんどのものでは新半細胞が旧半細胞と同じ長さに複元していた。細胞の幅および先端間の距離については各クローンで50標本を測微接眼レンズを用いて測定した。背側の湾曲度については顕微鏡写真より各クローンで20標本を測定した。

接合形態の測定は接合孢子形成後少なくとも10日を経た材料について行なった。孢子の長径と短径の測定は各系統で10標本に基づいたものであり、測定値にはトゲが含まれていない。トゲの長さは各孢子について比較的長いもの2個を選び、10個の孢子について測定した。

測定値については最大値、最小値、平均値、標準偏差、変異係数をもとめた (Tables 4-7)。

以下に論議されることがらは琉球の5系統 (25クローン)、愛知県の1系統 (1クローン)、北海道の1系統 (1クローン)、合衆国の3系統 (3クローン) に基づいている。

Sexuality については2系統において heterothallism が、他の8系統において homothallism が見られた。*C. calosporum* complex における heterothallism の例はこれまで知られていない。heterothallic の2系統は沖繩本島と西表島より得られたものであるが、両系統間でも接合孢子的形成が確認された。

10系統30クローンにおいて各クローンの変異係数は栄養細胞の幅において1.4-5.7%、先端間の距離において4.3-9.0%であった。宜名真産 (R-5) の10クローンは heterothallic であり、交配の結果同一の“生物学的種”に属するとみなされる。この10クローンよりランダムに選ばれた100標本の変異係数は幅において4.9%、先端間の距離では7.1%であった。これらの結果は今後野外における個体群の解析を行なう際の基礎資料となる。

与那覇岳山麓産 (R-9) の9クローンは栄養形態、接合形態および培養上の二、三の特徴において明瞭に区別される2系統よりなる。このうち大型の細胞を持つ系統は栄養細胞の幅において他の9系統とは不連続である (Fig. 1 参照)。この系統は栄養細胞の形態から *C. diana* EHRENBERG と *C. parvulum* NAEG. var. *maius* WEST とに最も近いが、両者の接合子が平滑であることから両者とは異なる生物に属するものとみなされる。小型の細胞を持つ系統は栄養細胞の先端の特徴において C-319, C-320, Akan-46 とよく一致し、また大きさと湾曲度の点でも互いに連続している。但し C-320 については細胞の幅と湾曲度の点で小型の細胞を持つ系統、Akan-46 の2系統とやや不連続であり、C-319 とは上記2点の他、幅と先端間の距離との比において大きく隔たっている。この4系統は KRIEGER (1935) による *C. calosporum* var. *maius* に含まれる。

残る5系統 R-5, R-11, A-2-22, C-318, R-12-2 のうち、C-318 は栄養形態において *C. calosporum* WITTROCK (1896) に最も近く、他の4系統と湾曲度において不連続である。栄養形態、接合形態から R-5 と R-11 との間には差違を認められない。これは交配の結果と一致する。上記2系統は栄養細胞の大きさに関しては A-2-22 と連続するが、先端の特徴と接合形態においては差違が認められる。R-12-2 は細胞の大きさにおいて他の9系統と不連続である。

本研究で用いた方法はミカヅキモのような特徴の少ない生物の分類学の発展に有効な方法であると考える。

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### Explanation of Plates 13-14

#### Plate 13

(Vegetative morphology)

A, C-319; B, C-320; C, Akan-46; D, strain R-9 with slender cells; E, strain R-9 with broader cells; F, strain R-5; G and H, R-12-2, G, giant cell; I, A-2-22; J, C-318.

#### Plate 14

(Sexual morphology)

A and B, strain R-5, MI at pH 8.0, B, abnormal zygosporangium with spines; C and D, strain R-9 with slender cells, CA, D, parthenospores with spines; E, C-318, uni-algal culture in PRINGSHEIM's soilwater medium; F, strain R-9 with broader cells, MI at pH 6.5; G and H, A-2-22, G, MI at pH 5.6, H, MI at pH 7.5; I, R-12-2, CA at pH 6.8.

