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Biosystematic Studies of the *Closterium peracerosum–strigosum–littorale* Complex IV. Hybrid Breakdown between Two Closely Related Groups, Group II–A and Group II–B*

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Since a pre-zygotic isolating mechanism has been shown to be functioning completely between Group II-B plus and Group II-A minus (Watanabe and Ichimura, 1978b), the reciprocal cross was investigated in order to clarify the presence of a post-zygotic isolating mechanism between the two sympatric closely related groups of the *Closterium peracerosum-strigosum-littorale* complex. Viabilities and fertilities of F_1, F_2 and backcross progenies of crosses within and between the two groups were studied using two representative pairs, IB–4–2 and IB–4–9 of Group II–A and KAS–4–30 and KAS–4–29 of Group II–B. Viability was estimated by % survival of isolated gones. Viability of F_1 progeny was 31.7% in the intergroup cross, while it was 70.0 and 46.0% in the intragroup cross of Group II-A and that of Group II-B, respectively. Viabilities of intragroup F_2 and backcross progenies were shown to be in the range of 32.0–76.0%. In contrast with this, those of F_2 and backcross progenies of the hybrids obtained in the intergroup cross were shown to be markedly reduced to the range of 0.0–2.5%.

Viable clones obtained in these intra- and intergroup crosses were almost all fertile, but one sterile clone was found among F_1 progeny of Group II-B. It was concluded that the so-called hybrid breakdown is also at work as an isolating mechanism between the two groups of this complex.

Key words: *Closterium* — Fertility — Hybrid breakdown — Post-zygotic isolating mechanism — Viability.

The two closely related groups of the *Closterium peracerosum-strigosum-littorale* complex, Group II-A and Group II-B, have been reported as maintaining their own morphological traits distinct from each other, although they are supposed to be of sympatric occurrence (Watanabe and Ichimura, 1978a). A pre-zygotic isolating mechanism (sexual isolation) has been reported to be functioning completely between Group II-B plus and Group II-A minus, but incompletely between Group II-A plus and Group II-B minus (Watanabe and Ichimura, 1978b). It has also been reported that

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zygospores obtained in these intergroup crosses can be germinated to form a "healthy" population of F_1 hybrids (Watanabe and Ichimura, 1978a).

In this paper, we have attempted to elucidate the presence of a post-zygotic isolating mechanism between these two closely related groups by investigating viabilities and fertilities of F_1, F_2 and backcross progenies of crosses within and between them.

Materials and Methods

The two pairs of complementary mating type clones used in this study were IB-4-2 and IB-4-9 representing Group II-A and KAS-4-30 and KAS-4-29 representing Group II-B (cf. Watanabe and Ichimura, 1978a). For backcross and F_2 progeny studies, one pair of complementary mating type clones was selected from F_1 progeny: X5-29 and X5-30 from IB-4-2 ×IB-4-9 cross; X6-8 and X6-7 from KAS-4-30×KAS-4-29; and X4-63 and X4-64 from IB-4-2×KAS-4-29.

The culture media and conditions and the mating methods are the same as those described previously (Ichimura, 1971: Ichimura and Watanabe, 1974). Treated by the zygospore germination method of Watanabe and Ichimura (1978a), zygospores obtained in the present experiments germinated generally within a few days after being rewetted with fresh C medium. Both of the two gones, young small closterioid cells, which emerged from a single zygospore were isolated individually into test tubes containing 10 m*l* of C medium. After one month of cultivation, numbers of test tubes containing a healthy clonal culture from a viable gone and numbers of those containing no vegetative cell or only a few unhealthy ones from an inviable or weak gone were counted. In this way, viability of progeny was estimated by % survival of isolated gones. Fertility and mating types of surviving clones were tested by crossing with each of the four test clones, namely the two representative pairs of Group II-A and Group II-B.

Results

Viability and fertility of F_1 progeny

At germination of a zygospore, normally in the genus *Closterium*, only two non-sister nuclei of the four meiotic products survive and a pair of two young vegetative cells appear from a germination vesicle which has been released out of an old resistant zygospore wall (Fig. 1). Viabilities of F_1 progeny of intra- and intergroup crosses were tested by isolating such pairs of F_1 gones from single zygospores obtained from crosses within and between Group II-A and Group II-B. The results are shown in Table 1, with the results of fertility tests of surviving F_1 clones. In intragroup crosses, viabilities of F_1 progeny of Group II-A and of Group II-B were 70.0 and 46.0%, respectively. The latter value was not as high as an expected one for an intragroup cross (cf. Discussion). On the other hand, viability of hybrid F_1 progeny of intergroup cross (IB-4-2×KAS-4-29) was 31.7% which was significantly lower than those values for intragroup crosses.

Perfect fertility was recognized in all surviving F_1 clones of these intra- and

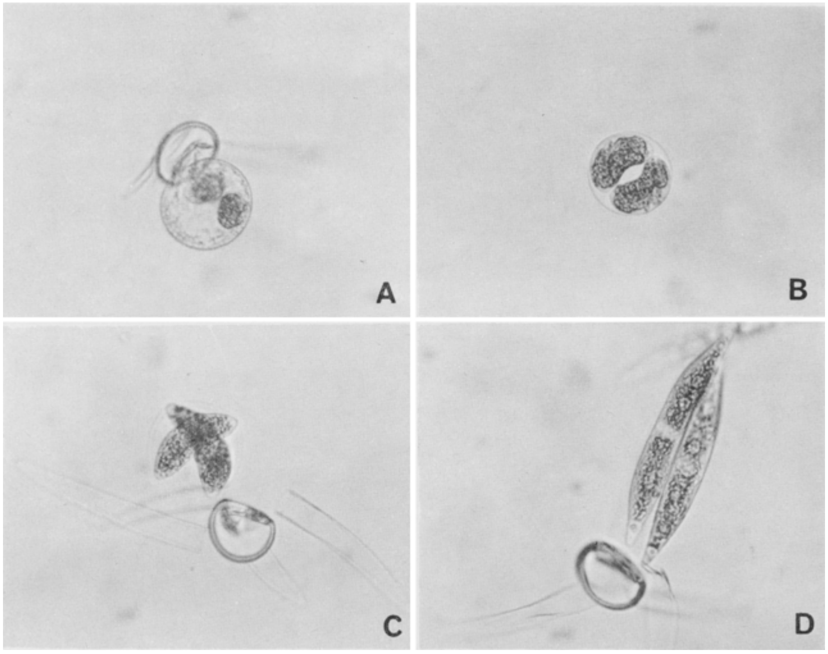

Fig. 1. Germination processes of zygospores in the *Closterium peracerosum-strigosum-littorale* complex which are exemplified by a pair of Group II-B. ×400. A, Released germination vesicle with empty zygospore wall; B, Two haploid gones in germination vesicle; C, Enlarging gones; D, Two young vegetative cells.

intergroup crosses, excepting one sterile F_1 clone of Group II-B intragroup cross. Mating types appeared to segregate in a normal 1:1 ratio among F_1 progenies of these crosses. Since pairs of two gones from single zygospores were isolated discriminately from those of others in this study, the fate of two gones as to their viability and mating type inheritance could be analyzed in detail, as shown in the lower part of Table 1. In the Group II-A intragroup cross, both of the gones tended to survive much more frequently than in Group II-B intragroup and Groups II-A×II-B intergroup cross, whereas, at a higher frequency, only one gone survived in Group II-B intragroup cross and both of the gones failed to survive in the intergroup cross. As to mating type inheritance, among 77 pairs of surviving clones in Group II-A intragroup cross, the two gones were of opposite mating type in 64 cases, whereas in the remaining cases both gones gave rise to clones of the same mating type. Non-sister nuclei of the second division of meiosis survive, which seems to indicate that the mating type locus segregates at the first division. According to Starr (1954) and Hamada *et al.* (1982), the centromere distance of mating type locus could be estimated as 16.9 map units from the data mentioned above, since the frequency of both gones being the same mating type is said to be dependent upon frequency of recombination between centromere and mating type locus. Although the numbers of surviving pairs were too small for this kind of genetic analysis in Group II-B intragroup and Groups II-A×II-B intergroup crosses, similar

Table 1. Viability and fertility of F_1 progeny

Cross	Intragroup		Intergroup[1)]
	Group II-A	Group II-B	Groups II-A×II-B
Plus parental clone	IB-4-2	KAS-4-30	IB-4-2
Minus parental clone	IB-4-9	KAS-4-29	KAS-4-29
Gones isolated	300	300	366
Gones survived	210	138	116
% survival	70.0	46.0	31.7
Fertility (%):	100	99.3	100
Mating type plus	108	69	57
Mating type minus	102	68	59
Sterile	0	1	0
Pairs isolated	150	150	183
Pairs survived:	77	25	26
% pair survival:	51.3	16.7	14.2
Opposite mating type	64	21	23
Both plus mating type	7	2	1
Both minus mating type	6	1	2
Sterile and mating type plus	0	1	0
Pairs with single survivor:	56	88	64
% single gone survival:	37.3	58.7	35.0
Mating type plus	30	43	32
Mating type minus	26	45	32
Pairs with no survivor	17	37	93
% none survival	11.4	24.6	50.8

1) The reciprocal cross, KAS-4-30×IB-4-9, yields very few zygospores (Watanabe and Ichimura, 1978a, b).

values could be calculated from the data shown in Table 1 as follow; 16.0 for the former and 13.0 for the latter case.

Viability and fertility of F_2 and backcross progeny

Using one representative pair of surviving F_1 clones from each of the intra- and intergroup crosses mentioned above, viability and fertility of F_2 and backcross progeny were studied in the same way. The results are shown in Table 2. Inasmuch as most of the zygospores obtained in these crosses were observed to germinate well to form two gones of morphologically normal appearance, no significant difference could be detected between intra- and intergroup crosses. However, a marked difference was recognized in % survival of isolated gones. Viabilities of F_2 and backcross progenies of Group II-A and Group II-B intragroup crosses were 64.0–76.0 and 32.0–44.0%, respectively, exhibiting the same values as those of the respective F_1 progeny. In contrast to this, viabilities of hybrid F_2 and backcross progenies were 0–2.5%, showing much lower values than that of the hybrid F_1 progeny which was significantly lower than those of intragroup F_1 progenies as already mentioned above. Thus, it was clearly shown that the so-called hybrid breakdown could operate effectively as post-zygotic isolating mechanism between the two groups in question.

It was confirmed that all of the surviving clones were fertile in F_2 and backcross generations in these intra- and intergroup crosses.

Table 2. Viability and fertility of F_2 and backcross progeny

Cross	Intragroup		Intergroup[1)]
	Group II-A	Group II-B	Groups II-A×II-B
Plus parental clone	IB-4-2	KAS-4-30	IB-4-2
Minus parental clone	IB-4-9	KAS-4-29	KAS-4-29
Plus F_1 clone	X5-29	X6-8	X4-63
Minus F_1 clone	X5-30	X6-7	X4-64
F_2 progeny			
Gones isolated	50	50	50
Gones survived	38	16	1
% survival	76.0	32.0	2.0
Fertility (%):	100	100	100
Mating type plus	21	9	1
Mating type minus	17	7	0
Backcross progeny of plus F_1 clone			
Gones isolated	50	50	40
Gones survived	33	20	1
% survival	66.0	40.0	2.5
Fertility (%):	100	100	100
Mating type plus	17	10	1
Mating type minus	16	10	0
Backcross progeny of minus F_1 clone			
Gones isolated	50	50	40
Gones survived	32	22	0
% survival	64.0	44.0	0
Fertility (%):	100	100	
Mating type plus	16	12	
Mating type minus	16	10	

1) The reciprocal cross, KAS-4-30×IB-4-9, yields very few zygospores (Watanabe and Ichimura, 1978a, b).

Discussion

The relationship between the two groups of the *Closterium peracerosum-strigosum-littorale* complex is very similar to that between Group A and Group B of *C. ehrenbergii* Meneghini reported by Ichimura (1981). In a natural field, these two closely related groups have never been observed to coexist in the same habitat. In laboratory experiments, however, they can be easily crossed to form a considerable number of zygospores which are capable of germinating. As is well known, aquatic microorganisms like *Closterium* are able to be transported passively from habitat to habitat by various means (cf. Maguire, 1963; Stewart and Schlichting, 1966; Proctor *et al.*, 1967; Atkinson, 1980). Therefore, it is highly possible for them to come into contact with each other in certain habitats.

It is of great importance to study the fate of hybrid zygospores obtained in laboratory crosses between these two closely related biological entities of sympatric occurrence. Recently Ichimura (1982) has shown that, in contrast to high (60–100)% survival values of intragroup F_1 progenies, extremely low (0–10)% survival values were recognized of F_1 progenies of most of intergroup crosses involving 15 plus and 12 minus clones of Group A and 10 each of the two mating types of Group B of *C. ehrenbergii*. He has also reported that abnormalities in zygospore germination as well as in mating type ratios of surviving hybrid F_1 clones were detected in all of the intergroup crosses of the same species. As mentioned in the text, no appreciable abnormality was recognized in germination of hybrid zygospores between Group II-A plus and Group II-B minus of the *C. peracerosum-strigosum-littorale* complex in F_2 and backcross, as well as F_1 generation. As to viability, although significantly lower % survivals were recognized in F_1 hybrids, still a considerable number of progeny could survive with the normal mating type ratio in F_1 generation of intergroup cross of this complex. These differences between *C. ehrenbergii* and the *C. peracerosum-strigosum-littorale* complex might suggest that the degree of genetic divergence between the two closely related groups is greater in the former than in the latter.

In Group II-B, rather low % survival values for intragroup crosses were obtained in F_1, F_2 and backcross progenies (Tables 1 and 2). This might be attributable to the pair of representative clones used in this study. One or both of them may have some unknown genetic factor(s) to decrease the values. Lippert (1967) has reported that % survival of F_1 progenies of six different pairs of *C. ehrenbergii* was in the range from 54 to 92%. Ichimura (1982) has also shown that similar values were obtained in intragroup crosses in *C. ehrenbergii* as mentioned above.

In contrast to F_1 generation, in the *C. peracerosum-strigosum-littorale* complex, it was shown that viabilities of F_2 and backcross progenies of intergroup hybrids decreased dramatically to an almost negligible level, whereas they remained at the same level as in F_1 generation in both Group II-A and Group II-B intragroup crosses. Thus, it can be said that hybrid breakdown in F_2 and backcross generation is operating strongly between Group II-A plus and Group II-B minus of this complex, whereas hybrid inviability in F_1 generation is at work effectively between Group A and Group B of *C. ehrenbergii* (Ichimura, 1982). In both of these two closely related sympatric groups of biological entity, substantial post-zygotic isolating mechanisms are prepared to operate in case of the breakdown of pre-zygotic ones such as sexual isolation and/or habitat preference.

In conclusion, it was further supported that the two groups of the *C. peracerosum-strigosum-littorale* complex are actually two different evolutionary units which are reproductively isolated from each other by various kinds of intrinsic isolating mechanisms. Discussion of their taxonomic status is, however, considered to be premature until such time as we have sufficient knowledge about many more organisms of this complex which are supposed to be distributed in other areas.

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