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Life history of *Halosaccion yendoi* I. K. Lee (Palmariales, Rhodophyta) and interspecific spermatium inoculation with *Palmaria* sp. from Hokkaido, Japan

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The palmariacean life history was demonstrated in laboratory culture of the marine red alga *Halosaccion yendoi* I. K. Lee, collected from Akkeshi and Muroran, Hokkaido. Attempts of intergeneric cross with *Palmaria* sp. from Muroran in the laboratory were made. The field-collected tetraspores of *H. yendoi* grew into macroscopic male gametophytes and microscopic female discs at a ratio of 1 : 1. After fertilization with field-collected spermatia of *H. yendoi*, the erect thallus of the diploid sporophyte developed from the carpogonium. The male gametophytes and sporophytes reached maturity in 5 months. Cytoplasmic fusion of spermatia with trichogynes in the intergeneric spermatium inoculation between *H. yendoi* and *P.* sp. resulted in nuclear fusion between the male nucleus from the fused spermatium and the carpogonial nucleus but no further development of the zygote was observed.

Key Index Words: gamete fusion—Halosaccion—*heterologous zygote—intergeneric cross—life history—nuclear fusion*—Palmaria—*Palmariaceae—reproductive isolation—Rhodophyta.*

The palmariacean algae exhibit a unique life history in which haploid tetraspores developed into macroscopic male gametophytes and into microscopic female gametophytes. Fertilization in these algae was observed between spermatia liberated from the male thalli and sessile carpogonia formed on the microscopic female germlings (van der Meer and Todd 1980, van der Meer 1981). Therefore, these algae can provide an excellent experimental system for studying the fertilization of red algae because it is easy to obtain a sufficient number of synchronously matured female gametes. Along the Japanese coast, the palmariacean life history has been so far reported only in *Palmaria* sp. (Deshmukhe and Tatewaki 1990 as *P. palmata*).

Among red algae, cross fertilization between different taxa or populations has been estimated by the degree of the development of carposporophyte after incubating female and male gametophytes in a common vessel under laboratory conditions. However, no study exists on the attachment, cytoplasmic and nuclear fusions in fertilization between heterologous gametes of red algae. Only one exception was the laboratory experiments on the attachment of spermatia to trichogynes between *Aglaothamnion neglectum* and other species of related genera (Magruder 1984).

Here, we have reported on the second Japanese species, *Halosaccion yendoi* I. K. Lee, which shows the palmariacean life history and, using this species and *P.* sp., we examined the attachment and fusion of gametes, and the fusion of nuclei in the fertilization between different genera.

Materials and Methods

Mature sporophytes and male gametophytes of *H. yendoi* were collected from intertidal zone at Aikappu, Akkeshi, Hokkaido on March 23-25, 1991, cast ashore at Tokkarisho, Muroran, on May 31, 1992, and *P.* sp. from Charatsunai, Muroran. They were

immediately transferred to the laboratory and kept thereafter under at a temperature of 10±2°C.

Tetraspores were harvested by agitating a small piece (ca. 2 cm×2 cm) of a fertile sporophytic thallus in 5 m*l* of filtrated seawater using a rotary shaker. After removal of thallus, the released spores were pelleted by hand centrifuge and suspended in 5 m*l* $ASP_{12}NTA$ (Provasoli 1963). This suspension was dispensed in 25 μl aliquots, each containing about 200 spores, on individual coverslips and incubated in a humid atmosphere 10±2°C under 45±5 $\mu E \cdot m^{-2} \cdot s^{-1}$, continuous light provided with cool-white fluorescent lamps.

After 5-8 days from spore inoculation, germlings was inoculated with spermatia by agitating in a 500 μl spermatial suspension for 10 min. The spermatial suspension was prepared by agitating a wiped small piece of fertile male gametophytic thallus in 5 m*l* of $ASP_{12}NTA$ for up to 1 hr. After spermatium inoculation, coverslips were then washed in $ASP_{12}NTA$ briefly and soaked in PES (Provasoli 1966) and incubated under cool-white fluorescent lamps at 35±5 $\mu E \cdot m^{-2} \cdot s^{-1}$, and a 14 : 10 light : dark photoperiod. The medium was changed once in every 2-4 weeks.

Intra- and interspecific spermatium inoculation between *H. yendoi* from Akkeshi and *P.* sp. from Muroran was carried out by agitating a coverslip with 5-day-old tetraspore germlings in 500 μl of spermatial suspension for 15 min. Coverslips were then washed by pipetting 10 times with ca. 3 m*l* of medium to remove loosely entangled spermatia. Beginning at the onset of spermatium inoculation, specimens after 180 min were fixed in 1% glutaraldehyde in seawater and stained with 0.5 $\mu g \cdot ml^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI, Sigma) in seawater for observation of the spermatial nuclear state. Photographs were made with Kodak Tri-X film. A comparative experiment was conducted with coverslips of tetraspore germlings derived from a single tetraspore suspension and aliquots of a single spermatium suspension were simultaneously inoculated.

Specimens for DNA microspectrofluorometry were prepared almost completely according to Goff and Coleman (1984) and Deshmukhe and Tatewaki (1993). Samples were fixed in Carnoy's fixative (3 : 1 mixture of 99.5% ethanol and glacial acetic acid) for more than 1 hr at 4°C, washed in running tap water for 1 hr, soaked in 50% (w/v) aqueous chloralhydrate for more than 2 hr, and squashed on uncoated coverslips and glass slides. Squashed samples attached to coverslips and glass slides were then dehydrated through alcohol series from 70 to 100% ethanol and air-dried. Dehydrated specimens were rehydrated in 200 mM KCl for 5-10 min and stained with 0.5 $\mu g/ml$ DAPI in McIlvaine's citrate buffer (pH 4.1).

The DNA fluorescence of nuclei was measured by Olympus OSP-1 system equipped with a MF450W band pass filter in addition to the filter combination used in fluorescence microscopy. An excited nucleus was quickly positioned inside the No. 5 pinhole (15 μm in diameter through a 100× objective lens) and the emission from the nucleus was measured. Non-nuclear areas of 15 cells of a similar type were measured as the background and the average intensity was subtracted from each nuclear fluorescence.

Results

Life history of H. yendoi *in laboratory.*

Field-collected tetrasporophytes formed the palmariacean tetrasporangia in the cortex with a distinct stalk cell (Fig. 1). Tetraspores liberated from these sporophytes were about 12-14 μm in diameter (Fig. 2) and began to divide 1-2 days after being attached to the coverslips. The first division occurred uniformly in the attached spores. However, their subsequent development were different between the two groups of germlings segregated at a ratio about 1 : 1 (Fig. 3).

One was the female germling that formed a trichogyne from a carpogonium, one of the derivative cells of the first division (Fig. 4). Another derivative cell continued to be divid-

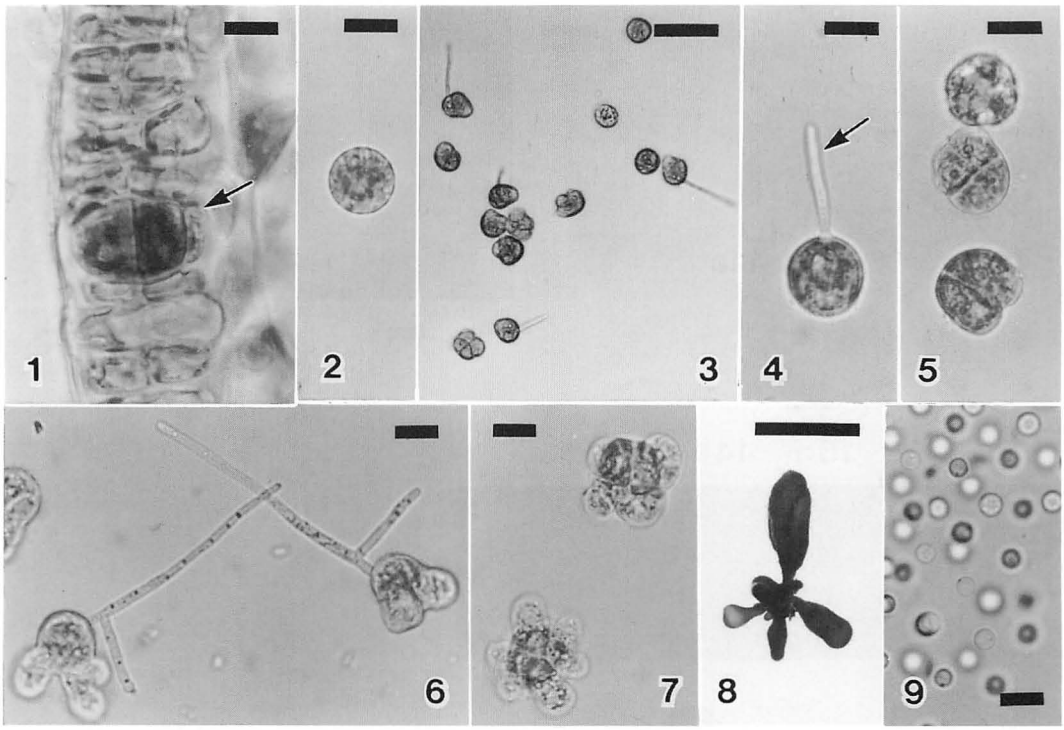


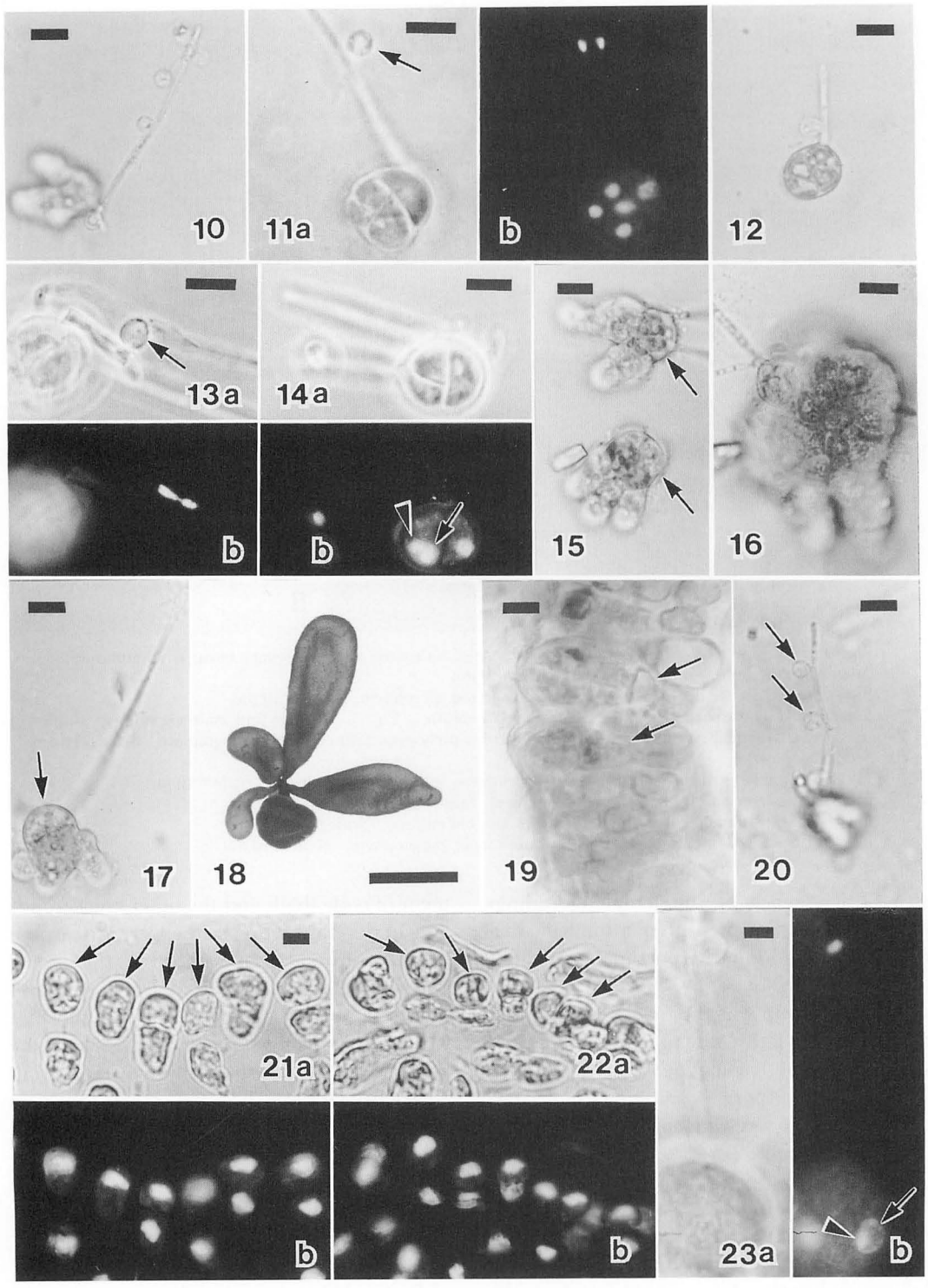
Fig. 1. Cross section of field-collected sporophyte of *Halosaccion yendoi* showing a tetrasporangium formed in the cortex. Arrow indicates the stalk cell. Scale=10 μm.
Fig. 2. Tetraspore liberated from the field-collected sporophyte. Scale=10 μm.
Figs. 3–5. Tetraspore germlings in a 4-day-old culture. Fig. 3. Female and male germlings. Scale=50 μm. Fig. 4. Female germling. Arrow indicates a trichogyne formed on a carpogonium. Scale=10 μm. Fig. 5. Male germlings. Scale=10 μm.
Fig. 6. Female germlings with branched trichogyne in a 6-day-old culture. Scale=10 μm.
Fig. 7. Male germlings in a 6-day-old culture. Scale=10 μm.
Fig. 8. Mature male gametophyte in a 5-month-old culture. Scale=10 mm.
Fig. 9. Spermatia liberated from the cultured male gametophyte. Scale=10 μm.

ed. Trichogynes of the female germlings often became branched after a long incubation (Fig. 6) and some carpogonia formed two trichogynes.

Another group was the male germling that developed into a disc due to the constant division forming no trichogyne (Fig. 5). After 5 days following spore inoculation, most germlings of both types developed into multicellular thalli having 2-5 prostrate projections (Figs. 6, 7).

The male germling developed erect, oblong thallus from a small prostrate disc. During the 2-month-culture, the erect fronds became 1-3 mm in length. Fifteen erect fronds on the coverslips, not inoculated with spermatia, were picked up for observation of the development of male thalli and the other erect fronds and undeveloped prostrates were discarded. The erect frond became saccate as they grew, and all of them became sexually mature in 4 months and began to liberate spermatia (Fig. 9) morphologically similar to those from the field-collected male gametophytes. During laboratory culture, the erect, saccate thalli of male gametophytes were grown to 5-10 mm in size when they reached to maturity (Fig. 8).

Spermatia from field-collected male gametophytes were spherical, about 5 μm in diameter, and morphologically similar to those of *P.* sp. When introduced into 5-8 day-old germlings of field tetraspores, the spermatia were easily attached to the trichogynes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formed on the female germlings (Fig. 10). During the fertilization processes, spermatial nuclear division (Fig. 11), cell fusion (Fig. 12), invasion and migration of derivative male nuclei in the trichogyne (Fig. 13), and fusion between male nucleus and carpogonial nucleus were observed (Fig. 14).

Initial development of the zygote was not detected clearly because it was difficult to distinguish derivative cells from the zygote and those from neighbor vegetative cells of the female gametophytic tissue. However, several days after spermatium inoculation, large, pigmented cells were cut off from basal cell bearing a trichogyne fused with spermatium (Fig. 15), later forming a mass of the cells on the female germling (Fig. 16). This cell mass was not formed on unfertilized female discs for a month (Fig. 17). Several female discs with a cell mass was left on the coverslips after removal of all the other germlings by needles, and the subsequent development of the zygote was observed.

The cell masses formed on the fertilized female germlings continued to grow, developed an erect projection and became hollow as they grew. They became reproductive about 5 months after spore inoculation and began to liberate tetraspores from tetrasporangia developed in their cortex (Fig. 19). The vegetative and reproductive structures were similar to those of field sporophytes. The cultured sporophytic fronds were relatively larger than those of male gametophytes, 12–18 mm in size at the time of maturity (Fig. 18). Tetraspores liberated from cultured sporophyte germinated in a similar fashion to those from the field sporophyte. When introduced to these germlings, the spermatia from the cultured male gametophytes fused with the trichogynes of mature female germlings developed from the tetraspores in culture (Fig. 20).

The outermost cortical cells and their nuclei of cultured sporophyte were apparently larger in size than those of cultured male gametophyte (Figs. 21, 22). It is easy to distinguish these two types of cells even when squashed and prepared on the same coverslips. Therefore, relative DNA fluorescence was compared between these cells prepared together using no control cell for standard DNA fluorescence. As shown in Figure 24, measured nuclear DNA fluorescence indicates that the sporophytic outermost cortical cells in culture has twice the nuclear DNA quantity of the male gametophytic ones.

Cross fertilization between H. yendoi *and* P. *sp.*

Experiments of intra- and interspecific fertilization were conducted several times and

Figs. 10–14. Fertilization of female germlings cultured from field-collected tetraspores with field-collected spermatia. Scales=10 μm. Fig. 10. Attachment of spermatia to a trichogyne. Fig. 11. Nucleus of an attached spermatium (arrow) divided into two male nuclei before cytoplasmic fusion with trichogyne. DAPI-stained. a) Phase contrast. b) Epifluorescence. Fig. 12. Cytoplasmic fusion between a spermatium and trichogyne. Fig. 13. Invasion and migration of two male nuclei from a spermatium (arrow) into the trichogyne. DAPI-stained. a) Phase contrast. b) Epifluorescence. Fig. 14. Fusion between a carpogonial nucleus (arrow) and male nucleus (arrowhead). DAPI-stained. a) Phase contrast. b) Epifluorescence.

Fig. 15. Early development of zygote 3 days after spermatium inoculation. Large, pigmented cells (arrows) were cut off from the carpogonium. Scale=10 μm.

Fig. 16. Further development of the zygote 10 days after spermatium inoculation. A mass of pigmented cells was formed on the carpogonium. Scale=10 μm.

Fig. 17. Carpogonium not fused with a spermatium 10 days after spermatium inoculation. No further development of carpogonium (arrow) occurred. Scale=10 μm.

Fig. 18. Mature sporophyte in a 5-month-old culture. Scale=10 mm.

Fig. 19. Cross section of mature sporophyte in culture showing a tetrasporangia formed in the cortex. Arrows indicate stalk cells. Scale=10 μm.

Fig. 20. Cytoplasmic fusion between spermatia (arrows) liberated from cultured male gametophyte and the trichogyne of female germling of tetraspore liberated from cultured sporophyte. Scale=10 μm.

Figs. 21, 22. Outermost cortical cells (arrows) of *H. yendoi* in culture. Fixed and stained for DNA microspectrofluorometry. Scale (10 μm) in 21 applies also to 22. Fig. 21. Sporophyte. a) Brightfield. b) Epifluorescence. Fig. 22. Male gametophyte. a) Brightfield. b) Epifluorescence.

Fig. 23. Female germling of *Palmaria* sp. from Muroran inoculated with spermatia of *H. yendoi* from Akkeshi 3 hr after spermatium inoculation. DAPI-stained. a) Brightfield. b) Epifluorescence. A carpogonial nucleus (arrow) fused with a male nucleus (arrowhead). Scale=5 μm.

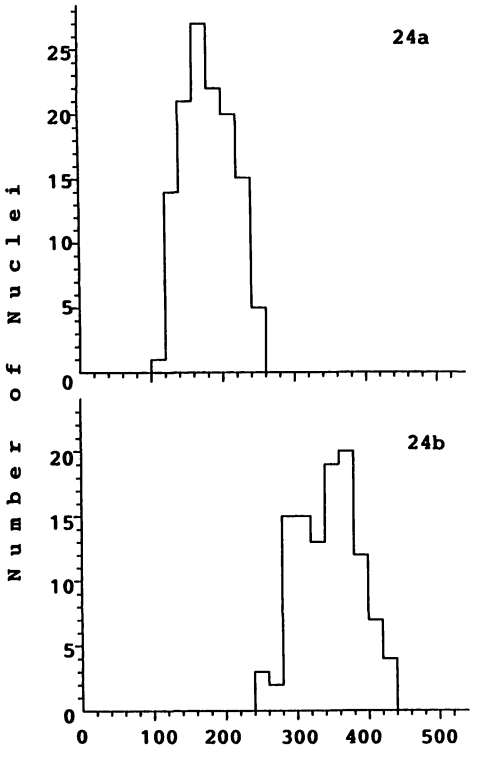

Fig. 24. Fluorescence values of DAPI-stained nuclei of outermost cortical cells of *H. yendoi* in culture. a) Male gametophyte. b) Sporophyte. The total number of measured nuclei (n), mean value ($\bar{x}$) and standard deviation (SD) are a) $n=110$, $\bar{x}=174.6$, SD=27.9. b) $n=110$, $\bar{x}=324.4$, SD=42.3.

results of a representative experiment are shown in Table 1. Data are presented as the numbers of trichogynes categorized into any one of the following three states: State 1) trichogynes to which no spermatium was attached; State 2) trichogynes to which at least one spermatium was attached but no cytoplasmic fusion occurred; and State 3) trichogynes fused with at least one spermatium. The occurrence of cytoplasmic fusion was judged by the invasion of a male nucleus from fused spermatium into trichogyne cytoplasm.

As shown in the table, although the ratios were invariably lower than those of intraspecific fertilization, up to 27% of trichogynes fused with the spermatia of the species of different genus. Fusions between caropogonial and male nuclei were observed in only a few carpogonia fused with spermatia even in intraspecific fertilization in *H. yendoi*. In the experimental specimen of the table, the nuclei in two carpogonia of *P.* sp. were observed to fuse with the male nucleus of *H. yendoi* (Fig. 23). On the other hand, despite the numerous male nuclei invading into the trichogyne cytoplasm, no fusion was observed between *Palmaria* male and *Halosaccion* caropogonial nucleus.

Some coverslips inoculated with spermatia of different species were subsequently incubated under the same condition used in the life history study. However, further division of the carpogonium and formation of the cell mass from carpogonia fused with spermatia was not observed after incubating for more than 2 weeks after spermatium inoculation.

Table 1. Intra- and interspecific spermatium inoculation between *H. yendoi* from Akkeshi and *P.* sp. from Muroran. State 1) No spermatium attached; State 2) Spermatium attached, but not fused; State 3) Spermatium fused.

Female	Male	Total number of trichogynes	Number (%) of trichogynes of		
			State 1	State 2	State 3
Halosaccion	*Halosaccion*	102	30 (29)	11 (11)	61 (60)
		108	30 (28)	8 (7)	70 (65)
Palmaria	*Palmaria*	108	57 (53)	16 (15)	35 (32)
		103	57 (55)	16 (16)	30 (29)
Halosaccion	*Palmaria*	111	87 (78)	8 (7)	16 (15)
		118	80 (68)	6 (5)	32 (27)
Palmaria	*Halosaccion*	108	91 (84)	15 (14)	2 (2)
		106	80 (76)	16 (15)	10 (9)

Discussion

The present study is a completion of the life history of *H. yendoi* in culture for the first time. *H. yendoi* exhibited a typical palmariacean life history where the tetraspores developed into macroscopic male and microscopic female gametophytes. Almost a similar life history was reported previously in other two species of the genus *Halosaccion* (van der Meer 1981, Mittman and Phinney 1985). The fertilization processes, e.g., spermatial nuclear division after attachment to the trichogyne, at random migration of male nuclei in the trichogyne, and cytoplasmic polyspermy, was also observed in *P.* sp. (data not shown).

No obvious development of unfertilized carpogonium existed at least for a month. However, the possibility of the development of haploid sporophyte from female disc, which was observed in *H. ramentaceum* cultivated in tank for a longer period (van der Meer 1981), could not be abandoned because different conditions in laboratory culture were adopted.

Using the two species, *H. yendoi* and *P.* sp., which exhibit the palmariacean life history, it is possible to carry out a comparative study on the attachment of spermatium to trichogyne in the fertilization of red algae between different taxa. The present results of the intergeneric spermatium inoculation show that the gamete attachment between different taxa was invariably lower than that of the intraspecific controls. An electron microscopic observation on the fertilization of *P.* sp. has revealed that covering of spermatium and trichogyne apparently mediate the attachment of these non-flagellated gametes (Mine and Tatewaki, unpublished observation). The difference in the gamete attachment may be related to the divergence in the biochemical nature of these covering materials between the two taxa. Studies on the adhesive nature of the covering of spermatium and trichogyne of the palmariacean algae were in progress in our laboratory.

Both cytoplasmic and nuclear fusion in the fertilization between species of different genera were readily observed in the present study. In contrast to this, no binding was observed between the gametes of *Aglaothamnion neglectum* (Ceramiaceae) and other species of related genera (Magruder 1984). Thus the stages where the fertilization between different genera is blocked are different between the Palmariaceae and Ceramiaceae. This difference may be related in part to the absence of any specialized appendage on spermatium in *Palmaria* and *Halosaccion*, which was reported in the species of the Ceramiaceae (Magruder 1984, Broadwater *et al.* 1991) and binds specifically to trichogynes (Magruder 1984).

In the natural population in Muroran and Akkeshi, *H. yendoi* and *P.* sp. grow on the common rocky shore and the male gametophyte and sporophyte of both species are reproductive simultaneously for several months. Therefore, it is probable that, as observed in laboratory, the cytoplasmic and nuclear fusion between the gametes of the two taxa occurs in the field population. However, since there was no cell division and cell mass formation from the carpogonia inoculated with the spermatia of different taxon in the laboratory, it is suggested to be impossible that the hybrid between *H. yendoi* and *P.* sp. exists in the natural population. In fact, no individuals which are regarded as the hybrid of these two genera in external morphology and anatomical feature have been found in the field.

Among the red algae, it is generally recognized that the development of interspecific hybrids is, if any, abnormal and often results in a sterile progeny (van der Meer 1988, p. 517). The experimental hybridization between the northeast and northwest Atlantic *Palmaria palmata* yielded intraspecific hybrids with greatly reduced fertility (van der Meer 1987). The developmental difficiency of heterologous zygote of the red algae may be due to the genomic incompatibility, and this assures the reproductive isolation between *H. yendoi* and *P.* sp. along the Hokkaido coast.

In contrast to the certain frequencies of gamete fusion between *H. yendoi* and *P.* sp., not a single fertilization was detected even in intrageneric crosses between Atlantic *P. palmata*

and *P. mollis* (van der Meer and Bird 1985). Though no crosses with species of different genera have been attempted in these Atlantic species, it is likely that fertilization would not occur with more remote taxa, i.e., species of the genus *Halosaccion*. Further investigations of the inter- or intrageneric fertilization with species of *Palmaria* of northeast Pacific or north Atlantic coast will reveal whether species differentiation in *Palmaria* was accompanied with barriers to heterologous fertilization at the level of gamete fusion.

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峯　一朗・舘脇正和：紅藻ベニフクロノリの生活史とダルスとの属間媒精

北海道室蘭および厚岸産のベニフクロノリ *Halosaccion yendoi* I. K. Lee の室内培養でダルス型の生活史が観察され，また室蘭産のダルス *Palmaria* sp. との属間受精実験が試みられた。天然の四分胞子は大型の雄配偶体と矮性の雌配偶体とに，一対一の割合で発生し，雌配偶体に形成された造果器は天然の不動精子と受精した後，複相の大型胞子体に発生した。雄配偶体と胞子体は 5 カ月以内に成熟した。*H. yendoi* と *P.* sp. という異なる属の間でも，受精毛と精子の細胞融合が起こり造果器核と雄核の融合も観察されたが，属間接合子の発達は見られなかった。
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