

ORIGIN OF APOMICTIC RED ALGAE: OUTCROSSING STUDIES OF DIFFERENT STRAINS IN *CALOGLOSSA MONOSTICHA* (CERAMIALES, RHODOPHYTA)¹

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Various red algae lack sexual reproduction and propagate by spore recycling, but it is still unknown how apomixis originates. In previous crossing experiments, we obtained an unusual hybrid of *Caloglossa monosticha* M. Kamiya through the outcrossing between a male from Australia and a female from Indonesia. This hybrid was morphologically identical to a normal tetrasporophyte, but its tetraspores grew into tetrasporophytes and repeated tetraspore recycling. During 5 years of culture, no sexual reproductive structures have formed on the tetrasporelings from this hybrid or its progenies. Further hybridization experiments revealed that all the five female strains from Indonesia successfully crossed with the male strain from the East Alligator River, Australia, and most of the F₁ sporophytes demonstrated tetraspore recycling, though the germination rates of these tetraspores were quite low. The ploidy level of the hybrid tetrasporophyte was similar to the normal tetrasporophyte, rather than the parental gametophyte, based on the comparison of relative DNA contents of their nuclei. Single strand conformation polymorphism (SSCP) and sequence analyses of the internal transcribed spacer 1 (ITS1) region indicated that the alleles from both parents were present in all the hybrid tetrasporophytes examined. These results suggest that this hybrid does not carry out meiosis during sporogenesis, and heterozygous diploid sporophytes arose from tetraspores. Therefore, we believe that obligate apomixis was generated through outcrossing between genetically different entities of *C. monosticha*.

Key index words: apomeiosis; apomixis; asexual reproduction; hybridization; mixed-phase reproduction; tetraspore recycling

Abbreviations: ITS, internal transcribed spacer; rfu, relative fluorescence units; SSCP, single strand conformation polymorphism

Most red algae show alternation of generations between haploid gametophytes and diploid sporophytes. After fertilization, the female gametophyte discharges diploid carpospores, and each carpospore grows into a tetrasporophyte. Meiosis usually takes place during tetrasporogenesis, so haploid tetraspores grow into male and female gametophytes. Besides this sexual life history, apomixis, reproduction without fertilization and meiosis, has been observed in various red algal taxa, and bispore/tetraspore recycling is relatively common in red algae, reported from nearly 40 genera (reviewed by Hawkes 1990, West et al. 2001). Each apomict seems to have evolved from its closely related sexual species based on their similar morphology, but there have been only a few investigations to demonstrate their close relationships (West and Zuccarello 1999, West et al. 2001, Zuccarello et al. 2005).

Some apomictic species may be obligate, completely lacking sexual reproduction, but facultative apomixis is a more common phenomenon in red algae (Hawkes 1990). In facultative apomixis, tetraspores mostly developed into tetrasporophytes but occasionally into gametophytes, which are reproductively fertile (Maggs 1988). Although red algal apomixis has been recognized for a long time and apomictic entities are much more dominant than sexual ones in some species (Maggs 1988), the causes of apomixis are still unknown.

The euryhaline red alga *Caloglossa* occurs in habitats ranging from marine to freshwater, preferably in brackish water, and many apomictic populations have been described in this genus (West et al. 1994, 2001). In a previous study, we performed crossing experiments using *C. monosticha* strains isolated from the East Alligator River, Australia, and Lombok Island, Indonesia (Kamiya et al. 2003). At that time, the F₁ sporophytes grew well, but their tetraspore-germlings were not fertile. Several months later, however, their tetraspore-germlings unexpectedly became fertile with tetrasporangial sori, not gametangial sori. These sporophytes have continued to discharge tetraspores and never produced sexual reproductive structures during 5 years of culture. This finding suggests that such an outcrossing may

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be one of the triggers to induce tetraspore recycling, but several questions still need addressing: (i) is this phenomenon reproduced by the same and/or different outcrossings, (ii) do all the progenies obtained from these outcrossings show tetraspore recycling, (iii) does meiosis, which is normally seen during sporogenesis, occur in this hybrid, and (iv) is this hybrid heterozygous?

In this study, hybridization experiments using several strains of *C. monosticha* from Australia and Indonesia were performed to check the reproducibility of this phenomenon. In addition, a number of tetraspore-germlings were isolated from the tetraspore-recycling hybrids, and the phase and reproduction of their next generation were examined to reveal the permanence of tetraspore recycling. Nuclear DNA contents of the hybrid sporophyte were compared with those of the normal gametophyte and sporophyte to elucidate whether meiosis does occur during sporogenesis. SSCP analysis, which is usually used to examine population structure by detecting point mutations, is also applicable for the investigation of nuclear inheritance among artificial hybrids (Coyer et al. 2002). We, therefore, carried out the SSCP analysis as well as sequence determination of the ITS1 region of the rRNA gene.

MATERIALS AND METHODS

Crossing tests. Methods of collection and culture are described in Kamiya et al. (1997) and West and Zuccarello (1999), and the strains of *C. monosticha* used in this study are listed in Table S1 (see the supplementary material). Nine male and 11 female strains isolated from Australia and Indonesia were used for the crossing tests (Fig. 1). One male isolate and one female isolate, each ~1 cm long, were placed in a 100 mL clean cup 90B (Risu Pack, Tokyo, Japan) containing 50 mL Provasoli's Enriched Seawater medium (Starr and Zeikus 1993) and cultured on a reciprocating shaker (30 cycles · min⁻¹) for 1–2 months, at room temperature. When carospores were discharged, they were transferred into a new plastic cup containing fresh medium and incubated without shaking.

Life cycle of F₁ hybrids and germination rate of their tetraspores. Two F₁ sporophytes were selected from the cross between the male strain from East Alligator River (MK1120, E. Alligator), Australia, and the two female strains from Lombok Island, Indonesia (JW3979, Lombok1 and JW3981, Lombok2). Twenty-

four tetraspore-germlings were randomly selected from each of the two sporophytes, and each tetraspore-germling was put into a 100 mL clean cup containing 50 mL medium. These were examined to determine whether each germling developed as a gametophyte or sporophyte and also to compare the germination rate of these tetraspores to that of tetraspores released from a normal sporophyte (hybrid between the male from Lombok2 and the female from Lombok1).

Ploidy determination. To check the ploidy level, nuclear DNA contents were compared among the hybrid tetrasporophyte (MK1120, E. Alligator X JW3981, Lombok2), normal tetrasporophyte (JW3981, Lombok2), and female (JW3981, Lombok2). Thalli ~5 mm long were fixed with 500 µL Carnoy's solution (100% ethanol:glacial acetic acid = 3:1) for 30 min at room temperature and then washed with 100% ethanol three times. The thalli were transferred into 100 µL saturated solution of trichloroacetaldehyde monohydrate (Wako, Osaka, Japan) and incubated for 1 h at room temperature. After rinsing with distilled water three times and once with PBS (13.7 mM NaCl, 268 µM KCl, 810 µM Na₂HPO₄·7H₂O, 147 µM KH₂PO₄, pH 7.4), the thalli were put on a glass slide, and 10 µL of 5X SYBr Gold (Invitrogen, CA, USA) in PBS was added. For infiltrating the stain into the cells, the thalli were treated in a microwave oven for 30 s. An extra 10 µL of 5X SYBr Gold was added, and the thalli were squashed with a coverslip, followed by incubation for 1 h at 4°C. Treated thalli were observed using an Olympus BX-51 epifluorescent microscope (Olympus, Tokyo, Japan) equipped with a U-MWIB3 filter that had an excitation of 460–495 nm and emission of >505 nm. Microscope images of stained nuclei were digitized using a 3-CCD RGB color camera (DP-70, Olympus) with resolution of 4080 × 3072 pixels. Fluorescence intensities of 84 nuclei and ambient backgrounds in the hybrid tetrasporophyte, normal tetrasporophyte, and female gametophyte were then analyzed with Lumina Vision software ver. 2.2 (Mitani Corporation, Fukui, Japan), and the value of each nuclear fluorescence intensity with its background subtraction was represented as the relative DNA content. It is known in many red algae that ploidy levels of nuclei change with the age of the cells (distance from apex; Goff and Coleman 1990) and that nuclear shape varies greatly, from discoid to reticulate. The nuclei we examined, therefore, were carefully selected based on their morphology (mainly round) and location (in pericentral cells or their flanking cells in the middle portion from the apex).

SSCP analysis and sequencing of ITS1. Heterozygosity of the apomictic strain was determined using the SSCP method. DNA was extracted from each strain (<100 mg wet weight [wwt]) by using E.Z.N.A. SP Plant DNA Miniprep kit (Omega Bio-tek, Doraville, GA, USA). PCR was carried out to amplify the ITS region between 18S and 5.8S rRNA gene (ITS1), ~250 bp long, by using 18F forward primer (5'-GAGGAAGGAGAAGTCGTAACA-3') and 5.8R reverse primer (5'-AGACATCCACCGTTACGAGTT-3'), both of which were newly designed. The PCR reaction was conducted in a total volume of 12.5 µL containing 50 ng of template DNA, 0.4 µM of each primer, 0.1 mM of each dNTP, 1× PCR buffer, and 0.25 U of TaKaRa ExTaq DNA polymerase (TaKaRa Bio., Shiga, Japan). The thermal profile for PCR was as follows: initial 1 min denaturation at 95°C, 30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 5 min. A portion of PCR sample (2.5 µL) was mixed with 7.5 µL of formamide-dye solution (5 mg xylene cyanol and 5 mg bromophenol blue in 5 mL formamide and 200 µL of 0.5 M EDTA, pH 8.0), denatured for 5 min at 85°C, and then cooled on ice. After pre-running for 30 min, 1 µL of each sample was loaded on a nondenatured 10% (acrylamide:bis = 39:1) acrylamide gel (TaKaRa Bio.) including 10% glycerol. Electrophoresis was carried out in 0.5X TBE (50 mM Tris, 41.5 mM boric acid, and 1 mM EDTA) at 0.01 A at 4°C for 4 h, by using an electrophoretic apparatus Miniprotein 3

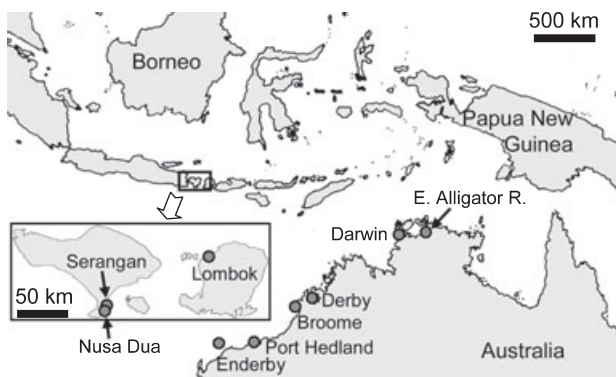


FIG. 1. Map of the collecting sites (gray circles).

system (Biorad, Hercules, CA, USA). The DNA bands were visualized using 1X SYBr Gold in 100 mL 0.5X TBE. For sequence analysis, each primary PCR product was cloned into the pGEM-T Easy cloning vector (Promega, Madison, WI, USA) in order to separate ambiguous sites among the differing intraindividual ITS copies. Five to 10 clones per amplification product were sequenced using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). These sequence data were deposited in the DNA Data Bank of Japan (DDBJ) sequence databases (accession numbers AB365320–AB365346).

RESULTS

In the crossing experiments, *C. monosticha* strains from Australia and Indonesia were reproductively compatible with each other, except for the strains from Enderby and East Alligator River, which were largely incompatible with other strains (Fig. 2). Such incompatible combinations frequently produced pseudocystocarps, which were smaller than normal cystocarps and did not form gonimoblasts, or formed carpospores that were released from cystocarps but did not germinate. However, the East Alligator River male that crossed with all the five female strains from Indonesia produced normal cystocarps (Fig. 3a), although the reciprocal crossings showed no reaction or produced only pseudocystocarps. Their carpospores successfully grew into tetrasporophytes (Fig. 3b), and tetraspores released from these tetrasporophytes became tetrasporophytes again, not gametophytes. These tetrasporophytes also produced a number of abortive

sporangia (20–40 μm), which were smaller than normal sporangia (55–75 μm) and were retained within the thalli without discharging tetraspores (Fig. 3c).

Mixed-phase entities with tetrasporangia and spermatangia appeared among normal tetrasporophytes in the crossings between the male strain from Palmerston Boat Ramp, Darwin, Australia (MK1125, Darwin2), and each of all Indonesian strains (Fig. 2). We did not check whether these spermatia and tetraspores on the mixed-phase entities were functional.

Germination rates of the tetraspores were examined in 24 tetraspore-germlings isolated from each of the two F₁ sporophytes, originating from the cross between the East Alligator River and Lombok1 strains (A01–A24) or Lombok2 strains (B01–B24; Table 1). Most of the germlings matured and discharged many tetraspores, all of which grew into tetrasporophytes again, but only one germling (B23 in Table 1) became a female gametophyte with many trichogynes. Although a majority of the tetraspores failed to germinate, and their germination rates were usually <10%, several sporophytes (A03, A05, A15, and B03) showed much higher germination rates (38%–57%; Table 1). In contrast, 143 out of 145 tetraspores from the normal tetrasporophyte (Lombok2 male × Lombok1 female) were germinated (germination rate = 99%).

Comparative nuclear DNA contents were examined in the hybrid tetrasporophyte, normal tetrasporophyte, and female gametophyte to check ploidy

		Indonesia					Australia					
		Lombok1 JW3979	Lombok2 JW3981	Serangan1 MK1313	Serangan2 MK1320	Nusa Dua JW3963	Broome MK1123	Derby MK892	Darwin1 MK1126	Port Hedland MK1139	Enderby JW4013	E. Alligator MK1120
female	male											
		Indonesia	Lombok2 JW3981	+	+	+	+	+	+	+	+	+
Serangan1 MK1313	+		+	+	+	+	+	+	+	+	+°	-*
Serangan2 MK1320	+		+	+	+	+	+	+	+	+	+°	-*
Australia	Broome MK1123	-	+	+	+	+	+	+	+	+	-*	-
	Derby MK890	+	+	+	+	+	+	+	+	+	-*	-*
	Darwin1 MK1126	+	+	+	+	+	-*	-*	+	+	-*	-*
	Darwin2 MK1125	+ ^m	+ ^m	+ ^m	+ ^m	+ ^m	-	-*	+	+	-*	-
	Enderby JW4013	-*	-*	-*	-*	-*	-*	-*	-*	+	+	-
	E. Alligator MK1120	tetraspore recycling	tetraspore recycling	tetraspore recycling	tetraspore recycling	tetraspore recycling	+°	-*	+°	+°	-*	+

FIG. 2. Result of crossing tests using *Caloglossa monosticha* strains isolated from Australia and Indonesia. + means F₁ sporophyte and subsequent F₁ gametophyte were fertile. +° means F₁ tetrasporophyte was fertile, but F₁ gametophytes were sterile. +^m means some of F₁ tetrasporophytes were mixed-phase with spermatangia, but others were normal. +* means cystocarps were produced, but carpospores did not germinate. -* means only pseudocystocarps, in which gonimoblasts do not develop, were produced.

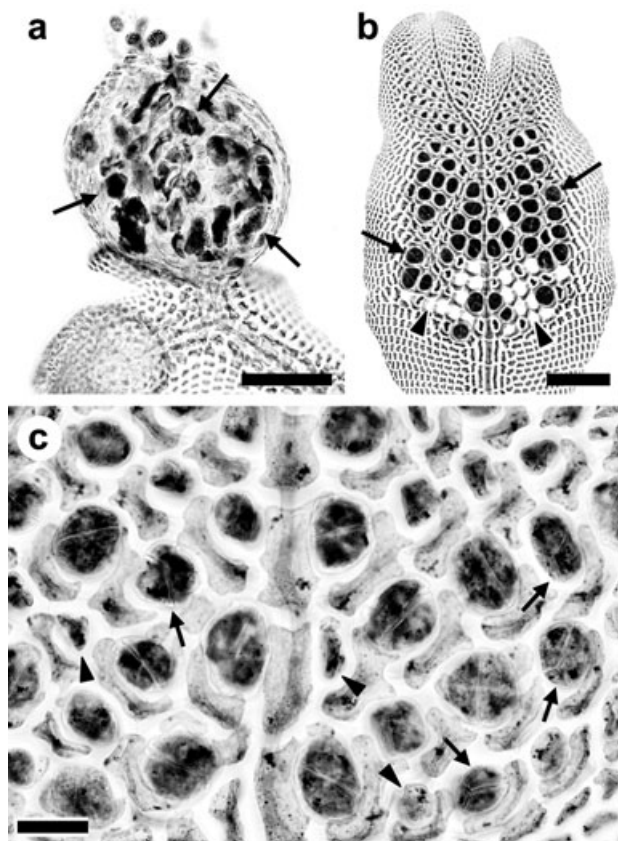


FIG. 3. Morphology of the hybrid between the male from East Alligator River and the female from Lombok2. (a) Cystocarp on the female from Lombok2. Many carposporangia (arrows) were in the cystocarp. Scale bar, 200 μ m. (b) Apical blade of F_1 tetrasporophyte producing many tetrasporangia (arrows). Some of the tetrasporangia were already released (arrowheads). Scale bar, 200 μ m. (c) Tetrasporangial sorus of F_1 tetrasporophyte. Many tetrasporangia were tetrahedrally or cruciately divided (arrows), but some sporangia appeared abortive (arrowheads). Scale bar, 60 μ m.

level changes (Fig. 4). The female had two peaks at 20 and 50 relative fluorescence units (rfu), whereas both the normal tetrasporophyte and hybrid strain showed an obvious peak at 50 rfu and a less distinct one at \sim 90–100 rfu.

SSCP analysis using the PCR product of the ITS1 region was carried out to detect the heterozygosity of the hybrid strain (Fig. 5). The number and position of the bands obviously differed between the male strain from East Alligator River (lane a in Fig. 5) and the female strain from Lombok2 (lane b in Fig. 5). Both paternal and maternal bands were recognized in their hybrid (lane c in Fig. 5). Subsequently, 12 tetrasporelings of the hybrid strains were analyzed using the same method, and all of them demonstrated the identical heterozygous band patterns (data not shown).

The ITS1 sequence was determined from clones of the male gametophyte from East Alligator River (five clones), female from Lombok2 (four clones),

TABLE 1. Germination rate of the tetraspores discharged from the two hybrid strains.

N	Spores ^a	Germlings ^b	Rate ^c (%)	N	Spores ^a	Germlings ^b	Rate ^c (%)
A01	179	5	2.8	B01	258	24	9.3
A02	13	1	7.7	B02	1,405	34	2.4
A03	34	20	59	B03	288	165	57
A04	1,526	116	7.6	B04	176	5	2.8
A05	39	15	38.5	B05	157	11	7.0
A06	462	15	3.2	B06	165	4	2.4
A07	554	45	8.1	B07	156	5	3.2
A08	176	15	8.5	B08	92	8	8.7
A09	163	13	8.0	B09	257	12	4.7
A10	252	0	0	B10	394	28	7.1
A11	281	0	0	B11	291	10	3.4
A12	452	26	5.8	B12	264	14	5.3
A13	313	6	1.9	B13	34	2	5.9
A14	363	15	4.1	B14	681	18	2.6
A15	97	51	52.6	B15	161	11	6.8
A16	312	10	3.2	B16	982	39	4.0
A17	312	15	4.8	B17	744	54	7.3
A18	628	8	1.3	B18	217	4	1.8
A19	345	13	3.8	B19	286	9	3.1
A20	237	3	1.3	B20	51	2	3.9
A21	1,317	63	4.8	B21	1,238	48	3.9
A22	56	3	5.4	B22	449	33	7.3
A23	1,022	10	1.0	B23	No data due to female		
A24	246	5	2.0	B24	168	26	15.5

This examination was carried out over \sim 2 weeks, so the number of released tetraspores differed among the germlings. A01–A24: tetrasporophytes isolated from the F_1 hybrid between the male from East Alligator River and the female from Lombok1. B01–B24: tetrasporophyte isolated from the F_1 hybrid between the male from East Alligator River and the female from Lombok2.

^aNumber of tetraspores discharged for \sim 2 weeks.

^bNumber of individuals germinated from the above tetraspores.

^cPercentage germination.

and their hybrids (18 clones). The sequence length of the ITS1 was 171–172 bp in the male and 164–165 bp in the female, and their genetic distance was 5.9%–6.6% based on the Tamura Nei model (Tamura and Nei 1993). The ITS1 sequences were slightly different within the male strain (one site change or one bp indel) and the female strain (one bp indel). Comparing 18 clones of the ITS1 sequence within the hybrid strain, nine clones showed identical or quite similar sequences to the male strain (up to 1.8% genetic distance), and eight clones were identical or quite similar to the female strain (up to 1.2% genetic distance). One remaining strain appeared to be a chimera; the upstream sequence was similar to the female, and the downstream sequence was similar to the male.

DISCUSSION

Recycling tetraspores unexpectedly appeared in the F_1 hybrid obtained from a previous crossing experiment (Kamiya et al. 2003). In this study, this phenomenon was reproduced in the cross between

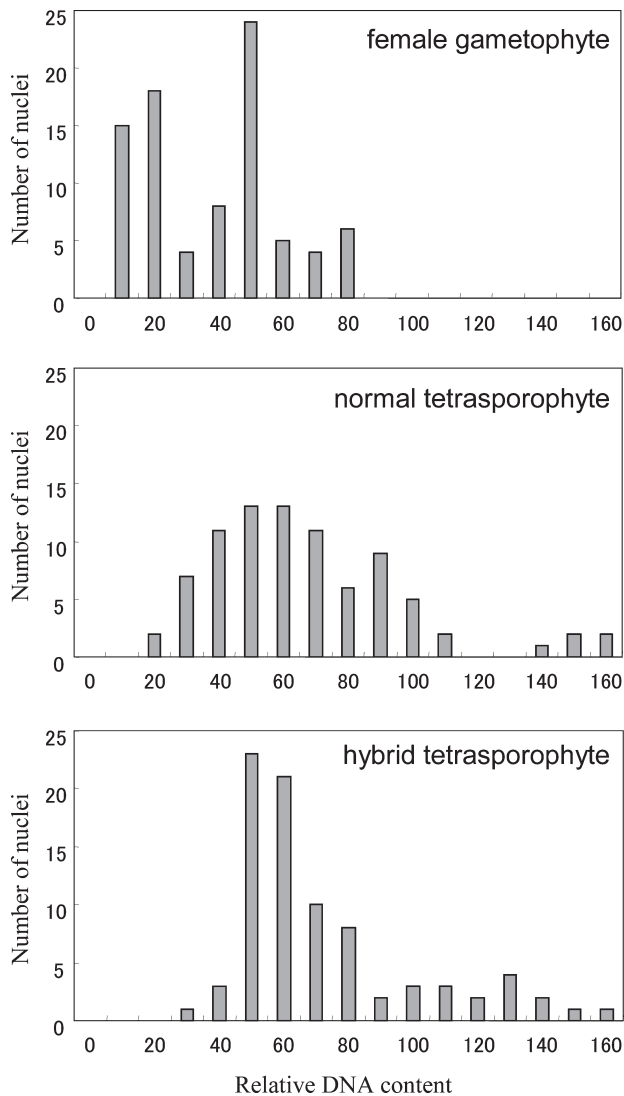


FIG. 4. Histogram to compare relative DNA content of each nucleus among three strains: the female gametophyte and normal tetrasporophyte from Lombok2, and the hybrid tetrasporophyte between the male from East Alligator River and the female from Lombok2.

the same pair, as well as in the crosses using a further four female strains from Indonesia (Fig. 2). As no reproductive reaction or formation of only pseudocystocarps was seen in the reciprocal crosses, reproductive isolation seems to have progressed between them. The molecular phylogeny inferred from LSU rRNA gene sequences also suggests that the Lombok1 strain (JW3979) is more closely related to the strain from Western Australia (genetic distance = 0.143%) than that from the East Alligator River (genetic distance = 0.645%; Kamiya et al. 2003). In contrast, all the Indonesian strains used in this study were isolated around Bali Island and were fertile with each other. In addition, we confirmed that the RUBISCO spacer sequence was identical between the female strains from Lombok1 and

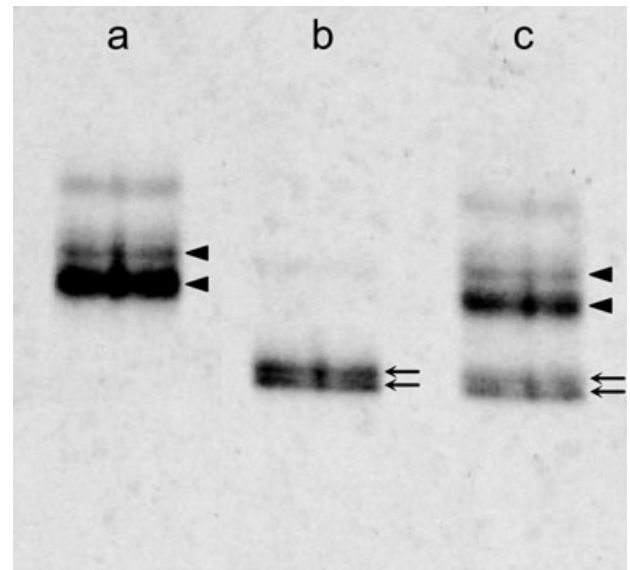


FIG. 5. Result of single strand conformation polymorphism (SSCP) analysis. Lane (a), the male strain from East Alligator River. Two bands (arrowheads) are visible. Lane (b), the female strain from Lombok2. Both bands (arrows) show different migration distance from those in the lane (a). Lane (c), the hybrid tetrasporophyte between the male from East Alligator River and the female from Lombok2. Among the four bands, the upper two bands (arrowheads) are identical to those in lane (a), and the lower two bands (arrows) are identical to those in lane (b).

Lombok2 (unpublished data). These results suggest that tetraspore recycling is inducible by the outcrossing between reproductively, as well as genetically, differentiated entities.

SSCP analysis clearly detected the different band patterns between the male and female strains and also the heterozygotic band patterns of the hybrid. Considering that 12 additional tetrasporelings of the hybrid sporophytes showed the same heterozygotic band patterns, it is highly possible that karyogamy takes place during the outcrossing event, and the resultant hybrid maintains both male and female genomes. In the ITS1 sequence analysis, heterogeneous sequences were detected within both male and female strains, and this may have resulted from base mispairs by *Taq* DNA polymerase (Keohavong and Thilly 1989, Huang et al. 1992). A chimeral sequence of the ITS1 between the male and female sequences was found from the hybrid strain, and this can also be an artifact. This phenomenon has been known as PCR-mediated recombination, which describes the process of in vitro chimera formation from related DNA template sequences present in a single PCR amplification (Meyerhans et al. 1990, Judo et al. 1998, Wu et al. 2007).

Apomixis is a general term for asexual reproduction without chromosomal reduction, in a broad sense, including parthenogenesis, agamospermy, and agamospory. The present SSCP and ploidy analyses suggest no chromosomal reduction in the

hybrid, although it is still uncertain whether meiosis occurs. If diploidization takes place before or after sporangial meiosis, the ploidy level of the resultant tetraspores could be identical to the parent. In this case, however, the homozygous genotype, as well as the heterozygous genotype, must be contained among the tetraspores. In this study, all the tetraspore-germlings from the hybrids were heterozygous, and furthermore, both maternal and paternal genotypes of the ITS1 were detected from the hybrid sporophyte. Consequently, it is considered that diploidization before/after meiosis does not occur in the heterozygous hybrid and that apomeiosis results in tetraspore recycling. It is known that more than one hundred copies of the ribosomal RNA genes, including the ITS region, are encoded in tandem on the same chromosome (Ganley and Kobayashi 2007). Because a heterozygous pattern of such multicopy genes can be also caused by chromosomal crossing-over (Vogler and DeSalle 1994), SSCP analysis based on a single-copy gene is necessary to confirm the absence of meiosis.

A number of culture studies had revealed spore recycling in various red algal taxa (reviewed in Hawkes 1990, West et al. 2001). Such a phenomenon, however, is frequently facultative; their spores grow into gametophytes as well as sporophytes during successive subcultures. For example, Maggs (1988) reported in *Atractophora hypnoides*, *Gloiosiphonia capillaris*, and *Schmitzia hiscockiana* that a majority of tetraspores recycled the crustose tetrasporophyte, but some developed into erect gametophytic thalli. In contrast, several species (or populations within a species) have been known to propagate only by spore recycling, which is called obligate apomixis (Hawkes 1990). The crustose coralline alga *Titanoderma pustulatum* var. *confine* (as *Dermatolithon litorale*) from the Swedish west coast produces no reproductive organs but uninucleate bispores, which are considered as apomeiotic (Suneson 1982). We have cultured the apomictic sporophyte obtained from the previous crossing test for 5 years, and no gametophyte has been reproduced from this strain so far. Although only one female gametophyte arose from 48 germ-lings of the two asexual strains in this study (Table 1), the other germ-lings became sporophytes and discharged tetraspores, all of which grew into tetrasporophytes again. This finding implies that obligate, not facultative, apomixis was induced by this outcrossing.

Although no apomictic specimens of *C. monosticha* have been found from nature, those of other species have been isolated from various populations. West et al. (2001) surveyed reproduction and life history patterns in culture of five *Caloglossa* species from Australia and New Zealand, and some isolates of *C. vieillardii* (as *C. leprieurii*) around South Australia gave rise to successive asexual generations of tetrasporophytes, while others showed normal alternation of generations. West et al. (1994) also examined life history patterns of *C. leprieurii* from

the Pacific Mexican coast and demonstrated that all the isolates from southern Baja California to the middle of the Pacific Mexican coast showed bispore recycling. These populations are northernmost at the eastern Pacific, and the nearest populations of sexually reproducing *C. leprieurii* are 1,200 km south of the southernmost asexual population (West et al. 1994). Recently, asexually reproducing strains of this species have been isolated from several other coasts of the western Atlantic and Indian oceans (J. A. West, unpublished data).

Apomixis is widespread and strongly associated with polyploidy in ferns (Lovis 1977, Wagner and Wagner 1980, Park and Kato 2003). Three-quarters of apomictic pteridophytes are triploid (Wagner and Wagner 1980), many of which are hybrids between diploid and tetraploid species (Gastony 1986, Park and Kato 2003). In *C. monosticha*, the apomictic hybrid demonstrated the same ploidy level to the normal tetrasporophyte, so the mechanism resulting in apomixis of this alga seems different from that in pteridophytes.

In flowering plants, apomixis is also frequently seen in polyploids, and apomictic plants usually produce both asexually and sexually derived embryos (Richards 2003). The components of apomixis comprise the absence of meiosis (apomeiosis), embryogenesis in absence of fertilization (parthenogenesis), and functional endosperm development (Ozias-Akins 2006). In addition, frequent occurrence of incomplete apomixis (facultative apomixis) is observed in many apomictic lineages of flowering plants, which indicates unstable regulation of apomixis. It is considered that apomixis and polyploidization are the outcomes of the temporal deregulation of normal sexual reproductive pathways, sometimes caused by interspecific hybridization (Praekelt and Scott 2001, Schranz et al. 2005). There is also some evidence that a certain epigenetic imprinting system could be altered to allow endosperm development in apomicts (Spielman et al. 2003). However, the genetic mechanisms underlying apomixis have not been determined, and apomictic mutants in sexual plants have never been isolated (Grossniklaus 2001, Spielman et al. 2003, Ozias-Akins 2006). Considering these aspects, the present alga may be a good organism in which to investigate apomixis because apomicts are easily inducible and the apomictic mechanism of this alga seems simpler than that of vascular plants.

Apogamic female gametophytes, which produce carpospores without fertilization, are known in some members of *Gymnogongrus* and *Mastocarpus*, and they are considered as obligate apomicts (Polanshek and West 1977, West et al. 1978, Guiry and West 1983, Masuda et al. 1984, 1987). Recently, Zuccarello et al. (2005) suggested that the apomicts of *Mastocarpus stellatus* around central Western Europe were generated through hybridization between the northern and southern breeding groups based on the

“mixed” pattern of their organellar genomes. Although the apogamic mechanism of *Gymnogongrus* and *Mastocarpus*, which has not been detected in other algae, is likely comparable with that of vascular plants, hybridization between reproductively differentiated entities may be common for generating apomixis.

The germination rates of tetraspores released from the present apomictic sporophytes were mostly lower than those of normal tetrasporophytes. Even if such apomicts were generated in nature, their low reproductivity may be compensated by many advantages of apomixis (Bilinski et al. 1989, Richards 2003). Formation of offspring, which is independent of fertilization in apomicts, seems easier and more reliable than in the case of related sexual plants. Apomixis is also advantageous where a normal life cycle cannot be completed (Suneson 1950) and can prevent undesirable gene flow (Spielman et al. 2003). Heterosis, the increase in such characteristics as size, growth rate, fertility, and yield of a hybrid organism over those of its parents, can be maintained through apomixis (Bilinski et al. 1989). We have no evidence of heterosis in the present apomicts, but recently, we obtained preliminary data that some apomictic sporophytes of *Caloglossa vieillardii* from Adelaide in Australia become fertile in lower temperature than normal sporophytes and gametophytes of the same species from Sydney (unpublished data).

In the present crossing tests, mixed-phase thalli possessing both tetrasporangia and spermatangia were generated from the crossing between the male strain from Palmerston Boat Ramp, Darwin (MK1125), and the female strains from Indonesia (Fig. 2), although it has not yet been examined whether their spermatia and tetraspores are functional. Mixed-phase reproduction has been reported in various red algal taxa, but there are few reports that mixed-phase algae were induced by such an artificial crossing (Van der Meer 1990). Interestingly, in most cases, mixed-phase algae produced male reproductive structures and tetrasporangial sori, rather than female with carpogonia and tetrasporangial sori (see West et al. 2001). Van der Meer (1990) proposed the mechanism to generate mixed-phase reproduction of *Gracilaria tikvahiae* by somatic recombination. According to his hypothesis, the sex-determining alleles are heterozygous in the normal diploid tetrasporophyte, and mitotic (somatic) recombination that generates homozygous sex-determining alleles gives rise to formation of (diploid) sexual reproductive structures on the tetrasporophytic thallus. A cue to clarifying the mechanism of phase/sex determination of *Caloglossa* may be found by further genetic investigation of the mixed-phase entities.

In conclusion, tetraspore recycling, one of the unusual life history patterns in red algae, originated from the crossing of *C. monosticha* strains isolated from northern Australia and Bali. Such apomictic plants may be generated in nature,

through genetic exchange between these two populations, because occasional long-distance dispersal of this algal group was suggested by biogeographic studies (Kamiya 2004). Many obligate apomictic strains of other *Caloglossa* species have been isolated worldwide, and they may also have originated from such outcrossings between genetically differentiated plants. If so, heterozygosity must have been fixed in these apomictic populations, and putative parents of each apomict can be identified based on their genotypes.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Strains of *C. monosticha* used in this study.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2008.00551.x>.

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