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Recent progress of molecular cytogenetic study on scleractinian (stony) corals

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Abstract

Chromosome study of scleractinian (stony) corals had been restricted to only counting chromosome numbers and measuring their lengths to delineate the chromosome types to establish their karyotypes for the past thirty years (before 2012), because chromosome preparations, which were made by the conventional squash-method, had not been suitable for molecular cytogenetic techniques. To advance the chromosome analysis of stony corals, it was necessary to utilize a molecular cytogenetic technique, such as fluorescence in situ hybridization (FISH) with DNA probes. We devised a newly improved method for suitable chromosome preparations made from coral embryos for FISH and made significant progress in molecular cytogenetic study of stony corals by FISH using our methods for making chromosome preparations. FISH made it feasible to carry out the establishment of their precise karyotypes, the gene mapping, FISH marker isolation and ascertainment of sex chromosome in stony corals. Molecular cytogenetics is useful for connecting and understanding the gap in classification between morphological and molecular (DNA sequences) analyses in the scleractinia. Therefore, better understanding of chromosome features may help to integrate the systematics of the data from both morphology and molecular sequences of stony corals. This review offers an overview of our research on molecular cytogenetics of stony corals; we are presenting our new findings on some stony corals using FISH, and overviewing former cytogenetic reports. The information provided here should be useful for the ongoing study of stony coral chromosome evolution, classification, genetics, and genome projects.

Key words: genomic hybridization, heterochromatin, karyotype, mini-satellites, rDNA genes

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INTRODUCTION

There have not been so many cytogenetic studies in Cnidaria (Anthozoa, Hydrozoa and Scleractinia), compared with those on other animals like mammals. Detailed cytogenetic study, like that done in mammals, is difficult because chromosome sizes of Cnidaria are relatively smaller than those of mammals. Fukui (1993, 1996) studied sea anemone chromosomes, Anthozoa, using conventional methods. Recently, Anokhin *et al.* (2010) studied *Hydra* chromosomes using molecular cytogenetic techniques. However, molecular cytogenetic study had not been carried out in Scleractinia before 2012. Stony corals are found throughout the world's oceans, including temperate and tropic intertidal zones. Most of these are found in shallow waters into which sunlight penetrates. It is estimated that there are more than 800 species of stony corals in the world (Veron 2000). However, their classification has been marked by confusion, because the data based on the methods used by conventional morphological traits (skeletal morphology) and by recent DNA sequence homology (ribosomal and mitochondorial DNAs) analysis do not always match up (Fukami *et al.* 2004).

Stony coral cytogenetics (chromosome study) is one essential way for charting relationships among stony corals in order to assist in coral classification. For studying chromosomes, the advent of molecular cytogenetics, such as fluorescence in situ hybridization (FISH), made it possible to infer alterations of gene order, syntenic homology among species, genetic isolation, speciation, evolution, and taxonomy in both plants and animals (Ansari *et al.* 2016, Dorritie *et al.* 2004). Before 2012, there were no reports on stony corals analyzed through chromosome bandings and molecular cytogenetic techniques, such as FISH. The technique of FISH using fluorescent DNA probes targeting specific sequences allowed researchers to draw homologies among the chromosomes of different coral species, yielding a wealth of new taxonomical information. Furthermore, FISH also allowed us to easily check whether species evolved by chromosomal fissions, autopolyploidy, or allopolyploidy.

At the beginning of our coral chromosome study (about 7 years ago), we adopted the conventional staining methods (using aceto-orcein, aceto-carmine or Giemsa) for studying and establishing karyotypes (chromosome morphology and numbers) of stony corals (Wijsman and Wijsman-Best 1973, Heyward 1985a, 1985b, Kenyon 1997, Flot *et al.* 2006). However, FISH analysis was not applicable on coral chromosome preparations made by the conventional squash-method. The detection of fluorescent labeled probes was interfered with innate florescence proteins. As the stony coral cytoplasm covered over chromosomes, the effectiveness of trypsin for the G-banding and that of $Ba(OH)_2$ for C-banding (Seabright 1973, Sumner 1978) were also not practical. To study coral chromosomes by FISH, we devised combined methods based on techniques for humans (Taguchi *et al.* 1993) and parasites (Hirai and LoVerde 1995). Our newly devised air-dried method for coral chromosome preparations made it possible to achieve the G- and C-banding patterns as well as FISH on stony corals. The molecular cytogenetic approach in combination with both morphological and molecular phylogenetics will hold promise for a greater understanding of stony coral relationships.

Here, we will review chromosomal study of the stony corals by molecular cytogenetic techniques which can assist in stony coral classification and help solve taxonomic issues.

STONY CORAL CYTOGENETICS IN THE PAST (BEFORE 2012) AND AT PRESENT (AFTER 2013)

Studies of stony coral chromosomes (Scleractinia, Cnidaria) in the past were limited and restricted to focus on their numbers and sizes (Heyward 1985a, 1985b, Kenyon 1997, Flot *et al.* 2006). Analysis of chromosomes made by the conventional squash method sometimes may mislead researchers concerning the numbers and sizes of chromosomes of stony corals, because chromosomes varied in size at the mitotic phases as well as with frequently having both an hsr (Taguchi *et al.* 2013, 2016, 2017) and/or secondary constrictions in one of the homologues, which did not match the length in between homologous chromosomes. As it was difficult to detect an hsr stained in conventional stainings, like aceto-carmine/orcein and Giemsa, precise karyograms of stony corals were not easily determined.

As shown above, before 2006, the way to observe stony coral chromosomes in mitoses was the traditional squash method using developing embryos around 12 hours, which were abundant in mitotic cells. Though the number of chromosomes was countable in a spread preparation of chromosomes by the squash method, not so many ideal mitoses for the counting of chromosomes could be easily obtained due to the overlapping of chromosomes. Furthermore, polyps of stony corals (somatic adult cells) were not suitable for cytogenetic studies, because it was difficult to find enough mitoses for analysis.

In the literature of coral chromosome studies, Heyward (1985 a, b) described the karyotypes of the four coral species *Goniopora lobata*, *Lobophyllia hemprichii*, *Montipora dilatata*, and *Montipora digitata*. Twelve years after the Heyward report, Kenyon (1997) studied 22 *Acropora* species and 5 other corals (4 *Montipora* and one *Fungia* species) concerning their number of chromosomes, but did not show their karyograms. Nine years after Kenyon's paper, Flot *et al.*

(2006) presented the chromosome numbers of six corals (four *Acropora*, one *Galaxea* and one *Favia*) in the proceedings of ICRS (10th International coral reef symposium). They reported that most of them have 28 chromosomes analyzed by a conventional staining.

In our laboratory, the method suitable for FISH was devised and applied on stony corals (Taguchi *et al.* 2013, 2014, 2016, 2017). So far, karyotypes of four stony corals were established using both conventional and FISH methods. The most important point in making preparations for bandings and FISH was to remove the cytoplasm over chromosomes while making chromosome spreads. Therefore, we applied the methods which were used for humans (Taguchi *et al.* 1993) and the parasite *Shistosoma* (Hirai and LoVerde 1995) with slight modifications.

The summary of cytogenetic data from the past and present are shown in Table 1. So far (before 2012), 36 species of corals have been analyzed by the conventional method without showing their karyograms. Most of the stony corals had 28 chromosomes in diploid. The species of *Acropora* had various numbers of chromosomes from 28 to 54 (Kenyon 1997). Flot *et al.* (2006) also found 26-40 chromosomes in *Acropora* and he found that one species *Galaxea fascicularis* (Oculinidae) has 26 chromosomes (2n). Interestingly, a variety of chromosome numbers were found mostly in *Acropora*. Chromosomal evolution might have happened more frequently in *Acropora* than in other families and it is suggested that genetic diversity happened in them, which supported the diversity of *Acropora* molecular data of former reports (Odorico and Miller 1997; Márquez *et al.* 2003). Therefore, chromosome numbers are an important index which contributes to the classification, especially in *Acropora* as their chromosome number varies among species.

Surprisingly, we found hsrs on three corals, *Coelastrea aspera* (Merulinidae), *Echinophyllia aspera* (Lobophylliidae), and *Platygyra contorta* (Merulinidae) though not in *Acropora*. An hsr seemed to be one of the crucial characteristics of stony corals with the exception of *Acropora*. However, *A. solitaryensis* was often observed slightly long pale portions of chromosome 1, like an hsr. An hsr could be detected in G-banded chromosome preparations which were treated to remove the cytoplasm over the chromosomes. It could be

Table 1. Cytogenetic data in scleractinian corals.

Family	Genus	No. of species*[1]	No. of Chromo.*[2]	Karyogram*[3]	hsr*[4]	References
Poritidae	*Goniopora*	1	28	+	N/O	Heyward 1985a
Acroporidae	*Montipora*	2	28	+	N/O	Heyward 1985b
Lobophyllidae	*Lobophyllia*	1	28	+	N/O	
Acroporidae	*Acropora*	22	28-54	-	N/O	Kenyon 1997
Acroporidae	*Montipora*	3	28	-	N/O	
Fungiidae	*Fungia*	1	28	-	N/O	
Acropolidae	*Acropora*	4	26-40	-	N/O	Flot *et al*. 2006
Oculinidae	*Galaxea*	1	26	-	N/O	
Faviidae	*Favia*	1	28	+	N/O	
Acroporidae	*Acropora*	2*[5]	30	+	?*[6]	Taguchi *et al*.
Lobophyllidae	*Echinophyllia*	1	28	+	+	2013, 2014,
Merulinidae	*Coelastrea*	1	28	+	+	2016, 2017
Merulinidae	*Platygyra*	1	28	+	+	

N/O: not observed,

*[1] Number of species studied.

*[2] Diploid chromosome numbers.

*[3] Most of the karyograms (karyotypes) are temporarily produced due to the uncertainty of their chromosome appearances (lengths and centromere locations).

*[4] hsr: homogeously staining region,

*[5] One of the *Acropora* (*A. pruinosa*) is the unpublished data.

*[6] Some were observed with having a relatively large portion of rDNA like an hsr.

difficult to find an hsr in the preparations made by the conventional squash method for chromosomes that were just stained by aceto-carmine/orcein or Giemsa. Figure 1 shows the representative G-banded karyogram of *Platygyra contorta* with a pale portion of the long arm of chromosome 12 which is an hsr (an arrowhead; one of the homologues). G-banding of stony corals, however, is not so clear as generally seen in that of mammals. Hsrs are chromosomal segments with various lengths and uniform staining intensity after G-banding (Fig. 1). This type of aberration is known as gene amplification (Biedler and Spengler 1976, Takaoka *et al.* 2012). Each chromosome 12 has a different length (one with an hsr seems longer than another), resulting in being classified into different homologues under conventional staining of squashed chromosome preparations.

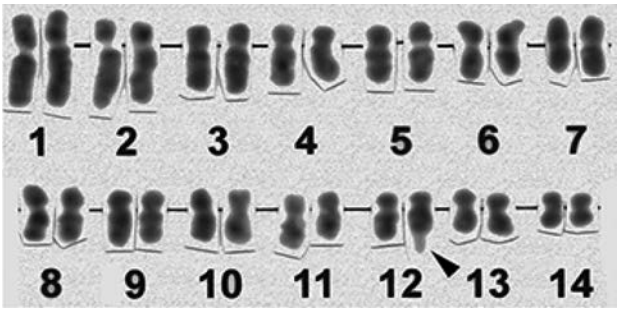

Fig. 1. Karyogram of *P. contorta*. The arrowhead indicates an hsr, which is palely stained by Giemsa (G-banding).

Chromosome types (Levan *et al.* 1964) of the corals studied were mostly metacentric and submetacentric, except *A. solitaryensis* (Table 2). The length of each chromosome was similar and smaller than those of humans and an unsatisfactory G-banding was obtained, which made it more difficult to establish their karyotypes precisely. One way to establish detailed karyotypes for each coral is to isolate more FISH markers such as painting probes and BAC library probes.

FISH ANALYSIS

Heterochromatin distribution patterns by genomic in situ hybridization (GISH).

Generally, heterochromatin largely consists of repeated sequences. If the distribution of repeated sequences over chromosomes are species-specific, it might be useful in distinguishing species. We attempted to find the distribution patterns of heterochromatin by GISH, because we discovered that GISH was a feasible method for detecting the distribution patterns of heterochromatin; GISH distinctly displayed the specific pattern of strong fluorescence signals on metaphase spreads. Specific hybridization patterns would be expected to be produced by the characteristic distribution of repeated sequences along chromosomes by GISH.

By GISH, we classified into the three distribution patterns of the repeated DNAs of corals and named, centromeric, telomeric and dispersive (both centromeric and telomeric) (Table 3). We showed the GISH images of four of five species (Fig. 2). GISH highlighted the centromere region on the chromosomes of *A. solitaryensis.* This may be a feature of *Acropora*, because we detected the centromere affinity of other species, such as *A. digitifera*, *A. hyacinthus* and *A. pruinosa* (unpublished data). Consequently, GISH analysis revealed that total genomic DNAs were usable to categorize five coral species into three patterns, which will assist the future taxonomic study of stony corals in regard to chromosome aspects.

Table 2. Chromosome types seen in 5 coral chromosomes.

Species	Chromosome number	Types*[1]			References
		Meta-centric	Submeta-centric	Acro-centric	
A. solitaryensis	30	6	22	2	Taguchi *et al*. 2014
A. pruinosa	30	4	26	0	Unpublished data
E. aspera	28	6	22	0	Taguchi *et al*. 2013
C. aspera	28	2	26	0	Taguchi *et al*. 2016
P. contorta	28	2	26	0	Taguchi *et al*. 2017

*[1] Three types were determined according to the centromere position (Levan *et al*. 1964).

Table 3. Heterochromatin distribution pattern.

Species	Distribution pattern	Fig. 2	Consensus sequences
A. solitaryensis	Centromeric	A, B, C	N/C
A. pruinosa	Centromeric	N/S	N/C
E. aspera	Telomeric	D, E, F	$(TTCCA)_n$
M. amakusensis	Dispersive *[1]	G, H, I	N/C
T. geoffroi	Dispersive *[1]	J, K, L	N/C

N/C: Not clarified.

N/S: Not shown in Fig. 2,

*[1] Dispersive type: mixed with centromeric and telomeric types.

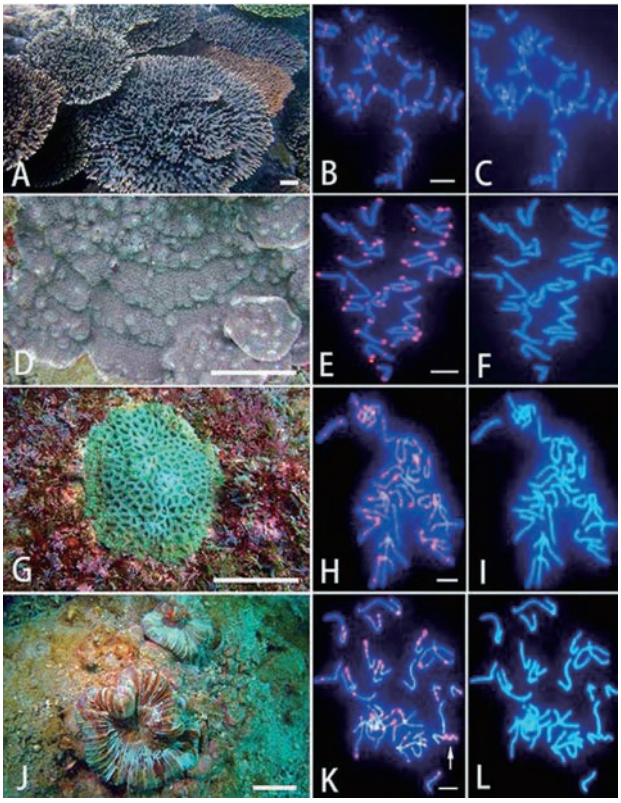

Fig. 2. The appearance of corals and heterochromatin distribution patterns**.** A, B and C: *A. solitaryensis* (centromeric), D, E and F: *E. aspera* (telomeric), G, H and I: *M. amakusensis* (dispersive), J, K and L: *T. geoffroi* (dispersive), B, E, H and K: FISH, C, F, I and L: DAPI (4', 6-diamidino-2-phenylindole) staining corresponding to FISH figures B, E, H and K, respectively. The bars in A, D, G and J indicate 5 cm. The bars in B, E, H and K indicate 5 μm.

As for repeated sequences, telomere sequences (TTAGGG)n in stony corals (Zielke and Bodnar 2010) are the same as those of humans (Vega *et al.*, 2003), coincidently. Therefore, we tried to find the common repeated sequences shared between stony corals and humans. The presence of specific heterochromatin consisted of consensus sequences (TTCCA)n was known as the motif of human satellite III DNA (Fowler *et al.* 1988). It was seen in the telomere region of chromosomes of *E. aspera* (Fig. 3A). Furthermore, it was surprising that the FISH probe from this heterochromatin (derived from the total genomic DNA) specifically hybridized with human chromosome 9 centromere (Fig. 3B) (Taguchi *et al.* 2013). This suggests that the common repeated sequences are shared between corals and humans. We also isolated the specific FISH marker derived human *Alu* sequences (Häsler and Strub 2006, Cordaux and Batzer 2009), which hybridized the specific loci on the coral chromosomes (Taguchi *et al.* 2016). Therefore, further study is ongoing to survey the distribution patterns of repeated DNA based on human satellites (Fowler *et al.* 1988, Jørgensen *et al.* 1992). The distribution of *Alu* repeats is also being examined on stony corals. These studies may help in the classification of stony corals.

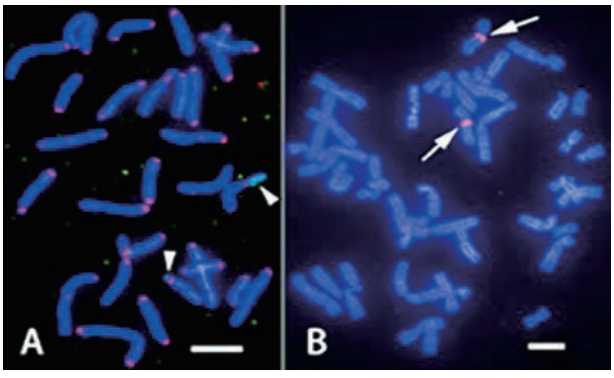

Fig. 3. Coral and human FISH images produced by the human satellite III motif DNA (TTCCA)n and rDNA probes**.** A: FISH with red heterochromatin (human satellite III motif DNA) signals and green rDNA (arrows) in the metaphase spread of *E. aspera*. Note that the longer one of the green signals (arrowheads) indicates an hsr. B: FISH with red heterochromatin signals in the human metaphase spread. Note that red distinct signals are seen on the chromosome 9 centromere regions (arrows) and red signals are reproduced by the same probe in both the coral and a human. Bars represent 5 μm.

Detection of rRNA gene, hsr, and sex chromosomes by FISH.

The whole genome DNA of the scleractinian (Cnidarian) species, *Acropora digitifera* (Scleractinia), has been sequenced (Shinzato *et al.* 2011) and the establishment of physical gene mapping on chromosomes (cytogenetic map) of stony corals is important for developing and promoting coral genetics and the genome project. Chromosomal mapping of DNA sequences by FISH has been applied to many organisms and enhanced to develop genome projects for several species, including mammals and plants (Levsky and Singer 2003). Therefore, obtaining FISH markers is not only crucial for physical chromosome mapping, but also profitable for establishing karyotypes in comparing syntenic homology and identifying chromosome changes (evolution) among coral species. However, there are few physical chromosome maps of stony corals at this point.

Many molecular studies of the rRNA genes have also been carried out on stony corals (McMillan 1989, Odorico and Miller 1997, Chen *et al.* 2000, Coleman 2008), because sequence studies of rDNAs regarding the conserved regions and the rapidly evolving regions (the so-called divergent domains or expansion segments) have been proved to be useful for investigating the evolutionary divergences that have occurred over the evolution of the metazoans (Hillis and Dixon 1991). Recently, we have succeeded in performing chromosome mapping of rDNAs by FISH on stony coral metaphase spreads from embryos. The loci of 5S, 18S, and 28S rDNAs were mapped on chromosomes of five corals (Table 4, Figs. 4 and 5). Different locations of rDNAs found in stony corals are inferred to correlate with cytogenetic events, such as chromosome alteration (evolution). As known in mammals, 5S rDNA was mapped on different loci from 18S and 28S rDNAs; for the first time, we were able to map the 5S rDNA on a chromosome of the stony coral (Fig. 5), the location of which was different from that of 18S & 28S rDNAs (Taguchi *et al.* 2017).

Chromosome mapping of rDNA by FISH revealed rDNA loci as well as the homogeneously staining regions (hsrs) from which rDNAs were derived (Taguchi *et al.* 2013, 2016, 2017). An hsr is one type of aberration in a chromosome's structure that is frequently observed in mammalian malignant tumor cells. In the region of a chromosome where an hsr occurs, a segment of the chromosome, that presumably contains a gene or genes which give selective advantage to the progression of the cancer, is amplified or duplicated many times. As a result of the multiple duplication, this chromosomal portion is greatly lengthened and expanded, and it is easily identified as an amplified region when chromosomes are stained with a fluorescent probe specific to the region by FISH (Takaoka *et*

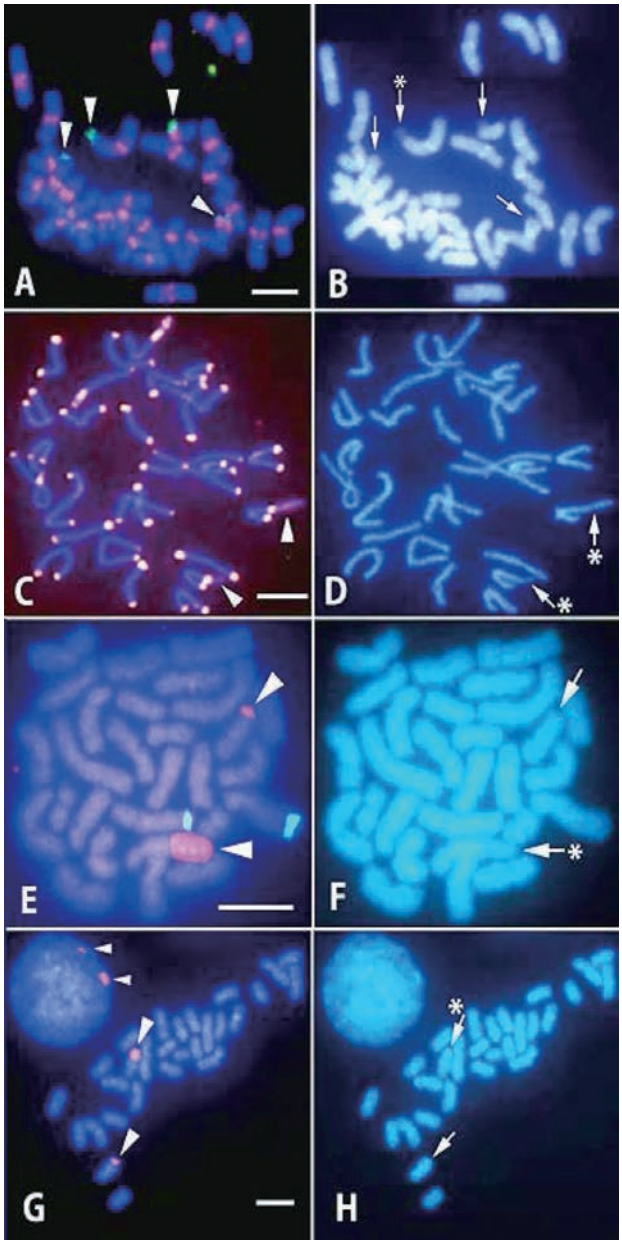

Fig. 4. Dual-color FISH images and their corresponding images stained with DAPI**.** A, B: *A. solitaryensis,* C, D: *E. aspera*, E, F: *G. aspera*, G, H: *P. contorta*, Arrowheads indicate rDNA loci (A, C, E and G) and domains in the interphase (G: small arrowheads). Arrows indicate the DAPI stained chromosomes with rRNA gene loci. Arrows with asterisks indicate the chromosomes with hsrs or hsr-like regions judged by FISH or DAPI staining. All red fluorescent signals indicate centromeres in A. Many yellow signals indicate telomeric hetero-chromachin which contains (TTCCA)n consensus sequences in C. Green signals on one of the homologous chromosomes derived from our isolated FISH marker in E (unpublished data).

Table 4. 5S, 18S and 28S rDNAs, hsr, and Y.

Species	Chromosomes (#) with rDNA genes				References
	18 & 28S rDNAs	5S rDNA	hsr *[1]	Y *[2]	
A. solitaryensis	#1 & #15	#6 *[3, 4]	-	+	Taguchi *et al.* 2014
A. pruinosa	#10	#5 *[3, 4]	-	+	Unpublished data
E. aspera	#13	N/D	+	-	Taguchi *et al.* 2013
C. aspera	#11	N/D	+	-	Taguchi *et al.* 2016
P. contorta	#12	#11	+	N/D	Taguchi *et al.* 2017

N/D: Not done.

*[1] hsr: homogeously staining region.

*[2] Y: presumed sex chromosome Y.

*[3] Unpublished data.

*[4] Some cells had a relatively large rDNA signals, which might be an hsr, detected by FISH.

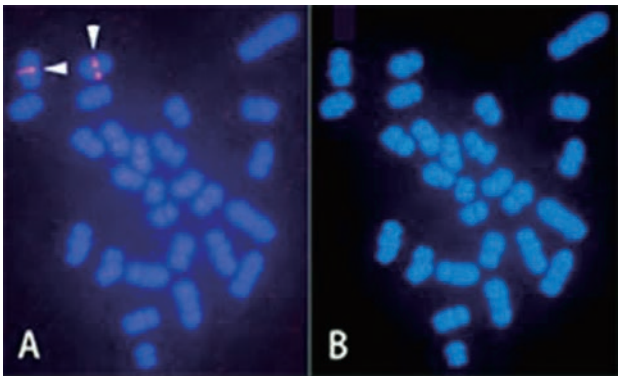

Fig. 5. Visualization 5S rRNA genes in *P. contorta.*
A: Cloned 5S rDNA (PC-T1) hybridized and highlighted on chromosome 11 centromere region (arrowheads). B: The same image counterstained with DAPI.

al. 2012). So, hsr regions found in stony corals consisted of mostly amplified rRNA genes, which was demonstrated by FISH. Using the probe which is reproduced through chromosome microdissection, we demonstrated that the hsr of this coral contained not only rDNA but also some other genes in C. aspera (Taguchi *et al.* 2016). It seems that an hsr is the characteristic cytogenetic feature of stony coral except *Acropora*, because we found hsrs in all five stony corals studied (*C. aspera*, *E. aspera*, *M. amakusensis*, *P. contorta*, *T. geoffroyi*) except *Acropora*, so far.

Furthermore, by comparative genomic hybridization (CGH, Fig. 6A) (Kallioniemi *et al.* 1992), the presence of probable sex chromosome was found in *A. solitaryensis* (Taguchi *et al.* 2014). Over-representation of green fluorescence indicates the DNA derived from sperm (Fig. 6B, an arrowhead). This chromosome seems to be one of the sex chromosomes like Y; the karyotype of this coral with this chromosome was possibly 30, XY. The presumable Y chromosome was seen in 14% of observed mitoses. This is the first observation of presumed sex chromosomes in stony corals (Taguchi *et al.* 2014). We also observed possible Y chromosomes in *A. pruinosa* (about 70% of metaphases; unpublished data). We need to survey other *Acropora* regarding the presence of sex chromosomes.

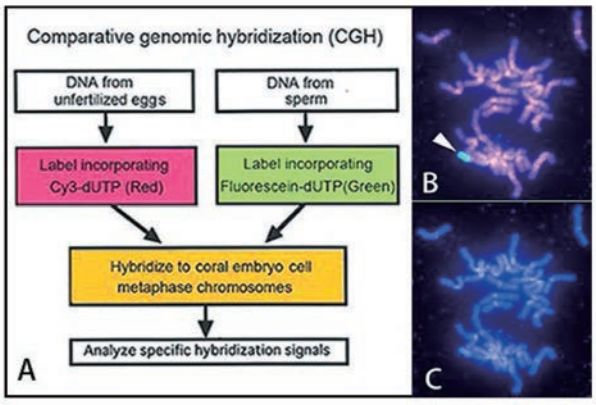

Fig. 6. Sex. A: CGH method which detects differences between sperm DNA and unfertilized egg DNA of *A. solitaryensis*. B: CGH image. Over representation of green signal (an arrowhead) indicates that it derives from sperm specific DNA. C: Counterstained by DAPI.

PERSPECTIVES

Cytogenetic study of stony corals began in 1973 (Wijsman and Wijsman-Best). The chromosome numbers of

thirty six stony corals were reported before 2006 (Table 1). However, no banding or molecular cytogenetic techniques were utilized for analyzing chromosomes in those reports (Heyward 1985a, 1985b, Kenyon 1997; Flot *et al.* 2006). Since 2012, we have been working on chromosomes of stony corals using banding and molecular cytogenetic analyses. Currently, we are accumulating the molecular cytogenetic information in stony corals. Molecular cytogenetic data have been successfully collected from four different species of stony corals. Followings are the novel cytogenetic characteristic features of stony corals; (1) an hsr, which mostly consisted of rDNA found in stony coral species except *Acropora*, (2) three patterns of the heterochromatin distribution over the chromosomes, which could be used as a taxonomic trait, (3) chromosome mapping of rDNA, which will assist in the coral taxonomy, (4) the presence of sex chromosomes in *Acropora*, at the least, such as *A. solitaryensis* (Taguchi *et al.* 2014) and *A. pruinosa* (unpublished data), (5) the specific FISH marker (PC-T1) related to 5S rDNA was isolated (Taguchi *et al.* 2017), (6) visualizing the telomere sequence by FISH.

Our "molecular" cytogenetic study has started on stony corals from 2010. Six years ago, the genome of *A. digitifera* (Scleractinian) was sequenced, beginning a coral genome project (Shinzato *et al.* 2011). As it becomes possible to perform FISH on stony corals, obtaining FISH markers for physical mapping (cytogenetic map) is important for the genome project. Now, we are trying to explore and collect the FISH markers systematically as much as we can. In the future, molecular cytogenetic data will help to advance genetics and develop coral taxa.

Overall, these new findings have led us to survey other unstudied stony coral species and look for more clues for solving the difficulties related to stony coral taxonomy and genomics.

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