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Genotyping the Clonal Population Structure of a Gorgonian Coral, *Junceella fragilis* (Anthozoa: Octocorallia: Ellisellidae) from Lanyu, Taiwan, Using Simple Sequence Repeats in Ribosomal Intergenic Spacer

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Chaolun Allen Chen, Nuwei Vivian Wei and Chang-Feng Dai (2002) Genotyping the clonal population structure of a gorgonian coral, *Junceella fragilis* (Anthozoa: Octocorallia: Ellisellidae) from Lanyu, Taiwan, using simple sequence repeats in ribosomal intergenic spacer. *Zoological Studies* **41**(3): 295-302. Asexual reproduction allows clonal genotypes to dominate a space and plays an important role in the ecology and evolutionary biology of many anthozoans. To analyze the contribution of clonality to local population structure requires genetic markers that can identify individual clones (genets). We explored the possibility of developing coral-specific PCR primers to amplify a region of tetranucleotide simple sequence repeats (SSRs, also known as microsatellites) in the ribosomal intergenic spacer (IGS) region. A primer set for PCR amplification of the IGS-SSR, A(C/G)(A/C)G, was designed and applied to investigate the clonal structure of 30 colonies of the gorgonian coral, *Junceella fragilis*, from Lanyu (Orchid Island) off southeastern Taiwan in 1999. In total, 12 scorable bands (length variants) and 2 distinct genotypes were identified. Of the colonies sampled, 66.67% represented a single dominant genotype. Low genotypic diversity revealed by several indices (G_0, G_0: G_E, and N_c / N) supports the scenario that the success of *J. fragilis* around Lanyu is likely due to vegetative propagation. The presence of 2 distinct genotypes sharing no common IGS length variants indicates that these 2 genotypes may have played the role of founders in structuring the Lanyu local population. The present study highlights the potential utility of IGS-SSR PCR techniques in delineating clonal diversity in *J. fragilis* populations on both local and global scales. http://www.sinica.edu.tw/zool/zoolstud/41.3/295.pdf

Key words: Microsatellites, *Junceella fragilis*, Genotypic diversity, IGS-SSR, Asexual reproduction.

Clonal structure has been previously assessed in anthozoans using techniques such as allozymes (Hoffmann 1976 1986 1987, Ayre 1983 1984, Hunter 1984 1993, Stoddart 1984, Willis and Ayre 1984, Ayre and Willis 1988, Burnett et al. 1995) and histocompatibility (Neigel and Avise 1983, Hunter 1985, Lasker and Coffroth 1985, Willis and Ayre 1985). However, for many species, these techniques do not provide genet-specific resolution, limiting analyses of the genetic structure of local populations (Stoddart et al. 1985, Willis and Ayre 1985).

Advances in molecular (DNA) technologies have provided alternative methods to measure the genetic relatedness of clonal anthozoans and the impact of local disturbance. Using minisatellite DNA fingerprinting, the clonal structure of the Caribbean gorgonian *Plexaura kuna* was revealed in detail (Coffroth et al. 1992, Coffroth and Lasker 1998). By combining these data with long-term demographic studies of *P. kuna* (Lasker 1984, 1990), a generalized model relating population structure to disturbance and mode of vegetative propagation was proposed for clonal species

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(Coffroth and Lasker 1998). However, several factors implicitly complicate the application of minisatellite techniques to other anthozoans. The presence of intracellular symbiotic dinoflagellates (zooxanthellae) causes significant cross-contamination in anthozoan DNA extracts which complicates the interpretation of band patterns on the autoradiographs (Coffroth et al. 1992). Furthermore, genomic DNA of high molecular weight is essential for the success of restriction digestion required in the minisatellite fingerprinting technique. This imposes major limitations on field-based DNA preservation methods (e.g., using ethanol or DMSO).

In order to overcome the limitations of minisatellite DNA fingerprinting techniques, the possibility of developing coral-specific PCR primers to amplify the ribosomal intergenic spacer region (IGS) and to assay its application in conjunction with restriction fragment length polymorphism (RFLP) analysis to detect the clonal structure of reef corals has been evaluated (Smith 1996, Yang 1996, Smith et al. 1997). IGS regions are the genic spacers between adjacent tandem repeats of the ribosomal RNA (rRNA) transcription units, and they accommodate complex regulatory sequences (polymerase binding, transcription regulation and termination, etc.). IGS regions show great variability in sequence and length both between and within species. Intraspecific IGS length variability is generally caused by different numbers of repeating motifs (reviewed in Gerbi 1985). IGS RFLP or sequence variation has been successfully applied in revealing patterns of descent in clonal lineages of fungi (Carbone et al. 1999) as well as population genetics and molecular biogeography of plants (Fukunaga et al. 1997, Ganley and Scott 1998, Koch et al. 1998, Barker et al. 1999). In the case of an agariciid coral, *Pavona cactus*, the level of variation detected by PCR-RFLP analysis of the IGS is surprisingly high; 17 distinct genotypes were detected among 28 samples collected within a fringing reef at Eclipse Island, suggesting that PCR-RFLP analysis of the IGS is a potential marker for clonal identification in scleractinian corals (Smith 1996). On the contrary, the IGS sequence of a gorgonian coral, *Junceella fragilis*, lacks repetitive motifs, although a set of tetranucleotide simple sequence repeats (SSRs, also known as microsatellites) is present in the putative 3'-end of the external transcribed spacer (ETS) (Chen et al. 2000a). When the SSRs are excluded from comparisons of *J. fragilis* IGS DNA sequences between geographically isolated populations (Taiwan vs. Australia), the sequences differ by < 4%, suggesting that the variability of IGS outside the SSR region is low in *J. fragilis* (Chen et al. 2000a). In addition, PCR-RFLP analyses of the IGS of *J. fragilis* using 30 restriction endonucleases showed only 2 classic RFLPs that were detected between geographically distant populations, but not within local populations (Yang 1996). On the contrary, the SSRs might indeed account for the major polymorphism within/between IGS regions or individuals of *J. fragilis* due to the characteristic of a high mutation rate for simple sequence repeat motifs (Estoup and Cornuet 1999).

In this study, we designed a set of specific primers to amplify the IGS-SSR of *Junceella fragilis* and assess the clonal population structure of *J. fragilis* around Lanyu, southeastern Taiwan. *Junceella fragilis* is a zooxanthellate and common seawhip on Indo-Pacific coral reefs, and has been reported from the Persian Gulf, Okinawa, the Philippines, Singapore, the Great Barrier Reef, and Taiwan (Chen and Chang 1991). Distribution of *J. fragilis* is usually patchy and clumped due in part to the short distances of fragment dispersal (i.e., asexual propagation) (Walker and Bull 1983, Vermeire 1994). PCR application of the IGS-SSR in *J. fragilis* provides the 1st example of the utility of tetranucleotide SSRs in delineating coral clonal population structure. The potential utility of IGS-SSR PCR techniques in delineating the clonal diversity in *J. fragilis* populations on both local and global scales is also highlighted.

MATERIALS AND METHODS

Sample collection

A total of 30 samples of *Junceella fragilis* was collected from Lanyu (Orchid Island), an island off southeastern Taiwan (21°58'N, 121°28'E), in 1999. Mapping or the transect line method is applied to avoid collecting clone mates during sampling of benthic anthozoans (Coffroth and Lasker 1998). However, *J. fragilis* usually occurs sporadically in patches at the reefal edge where sea currents are strong (Chen and Chang 1991). At Lanyu, *J. fragilis* was only found in 30-m-deep water at 1 diving site after an intensive scuba survey. For safety reasons, we did not apply the methods of Coffroth and Lasker (1998) to measure exact distances between samples. Instead, we swam downstream with the current and clipped the tip of each colony encountered at an eye-esti-

mated distance of about 2.5 m apart, which is about the body length of a diver with arms extended plus fins. This method allowed us to maximize the possibility of collecting different genotypes of *J. fragilis* in a local population. Samples were placed in a labeled bag and frozen in liquid nitrogen or dry ice for transfer to the laboratory. Samples were stored at -70°C prior to analysis.

DNA extraction, amplification, cloning, and sequencing

The DNA extraction protocol was modified from that of Chen and Yu (2000) and is described in detail in Chen et al. (2000a b). The complete sequence of the IGS clone with the flanking coding region of 28S and 18S rDNA was submitted to GenBank with the accession number AF154670 (Chen et al. 2000a). PCR was performed in a PC-9606 thermal sequencer (Corbett Research) using the following thermal cycle: 1 cycle at 95°C (3 min); and 30 cycles at 94°C (30 s), 55°C (1 min), and 72°C (1 min). The amplification reaction used 50-200 ng of template and BRL *Taq* polymerase in a 50-μl volume reaction, using the buffer supplied with the enzyme, under conditions recommended by the manufacturer. PCR products were electrophoresed in a 1% agarose (FMC Bioproduct) gel in 1x TAE buffer to assess the yield. Amplified DNA was extracted once with chloroform, precipitated with ethanol at -20°C, and resuspended in TE buffer. PCR products were cloned using the pGEM-T system (Promega) under conditions recommended by the manufacturer. Nucleotide sequences were determined for complementary strands of 5 clones from each sample using an ABI 377 genetic analyzer.

Genotyping

The PCR reaction was conducted with the conditions described above except that the final volume was reduced to 15 μl. The 15-μl reaction mixture contained 50-100 ng of template DNA, 0.5 μM of each primer, 80 μM of dNTP (dATP concentration 1: 10), 1.5 mM $MgCl_2$, 0.02 μl of [^{32}P]-dATP at 1000 Ci/mmol, and BRL *Taq* polymerase under conditions recommended by the manufacturer. After amplification, 4 μl of the solutions was mixed with 0.5 volumes of formamide loading buffers. Samples were heated (90°C, 2 min) before being run on a 6.5% sequencing polyacrylamide gel. The M13 sequence was used as a size standard. In order to test the repeatability of the multi-copy marker, such as the IGS-SSR, we conducted the PCR using genomic DNA obtained from 2 independent extractions of the same individual colony. The same banding pattern is expected from different extractions of the same colony if repeatability is high.

Genotypic diversity statistics

Although, strictly speaking, the IGS-SSR profile represents neither a single- nor a multi-locus DNA fingerprint, several indices of genotypic diversity can be calculated from the number of genotypes identified in the Lanyu population. First, N_c/N, the proportion of distinguishable genotypes, was used to examine genotypic diversity (Ellstrand and Roose 1987); where N_c is the number of genotypes (clones), and N is the sample size. When all individuals have an unique genotype, N_c/N will equal 1, whereas N_c/N approaches 0 when most individuals belong to a few clones. Second, observed genotypic diversity (Stoddart 1983b), G_0, was calculated as:

$$G_0 = \frac{1}{\sum_{i}^{k} gi^2};$$

where gi is the relative frequency of the i^{th} genotype, and k is the number of genotypes (Stoddart 1983b); gi is estimated by ni/N; and ni is the number of individuals of genotype i in a sample of N individuals. When there is only a single genotype, G_0 is a minimum of 1. When the numbers of genotypes are evenly distributed, G_0 will be a maximum of k. G_0: G_E, the ratio of observed genotypic diversity to the genotypic diversity expected in a strictly sexual population, can be used to assess the relative contribution of asexual propagation to a local population. G_E is an exact value and not an estimate with an associated variance as when using isozyme allele frequencies (Stoddart and Taylor 1988). Because PCR IGS-SSR can unambiguously identify genotypes (see "Results and Discussion"), the expected number of genotypes in a strictly outcrossed population would be N (the number of samples) as indicated by the DNA fingerprinting technique (Coffroth and Lasker 1998). Third, the number of individuals per clone (genotypic evenness) was calculated by G_0/N_c. When individuals are distributed evenly among clones, genotypic evenness has a maximum value of 1, but approaches 0 when a single

clone dominates the population (Coffroth and Lasker 1998).

RESULTS AND DISCUSSION

Tetranucleotide simple sequence repeats (SSRs), A(C/G)(A/C)G, at the 5'-terminus of the IGS were identified by visual inspection of the complete sequence of IGS in *Junceella fragilis* (Chen et al. 2000a). In the example shown as figure 1, compound sequences of SSRs, containing 16 repeats, are located between positions 344 and 408. In order to assess the variability of SSRs within the IGS region, the specific primer pair, junrept-5: 5'-GTC CCT TCG CCT CGT GAT GGC TTC GC-3' and junrept-3: 5'-AAT ATC GAG GAA TGT CGA CGG TCC-3' were designed (Fig. 1). These primers allowed PCR fragments to be amplified with sizes estimated to be above 100 bp.

A total of 12 scorable bands (length variants), ranging from 126 to 188 bp, could be detected in the polyacrylmide sequencing gel (Fig. 2). In order to determine the level of size homoplasy for each length variant, 50 PCR clones from 10 individual *J. fragilis* were randomly chosen and sequenced (Table 1). We confirmed that the same length variants amplified from different individuals are homologous, i.e., they have the same core SSR motif (Table 1). In addition, sequencing analysis also indicated that the occurrence of some length variants was due to insertions and deletions (indels) of the SSR motif, not by gains or losses of repeats. For example, the core sequence of the 126-bp variant is similar to that of the 128-bp variant, except for 2 indels at the final 2nd repeat (Table 1). Size homoplasy is one of the concerns when using SSR frequencies to estimate population parameters (Estoup et al. 1999). SSRs of the same size can arise from mutational events outside of the repeat by interrupting perfect repeat-producing variants (alleles) that are not identical by descent. For example, the effect of size homoplasy on the resolution of population structure has been demonstrated for bees and 1 freshwater snail (Viard et et al. 1998).

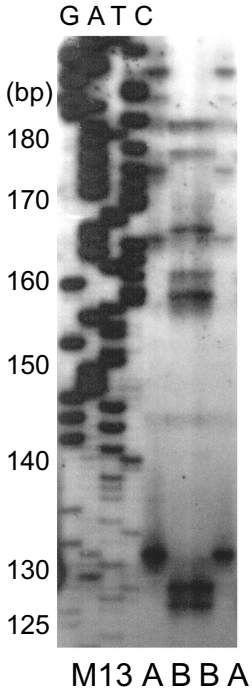

Fig. 2. IGS-SSR bands detected using the junrept primers from 4 colonies of *Junceella fragilis*. A size (bp) marker is indicated by the M13 sequence at the 4th lane from the left of the gel. Genotypes A and B are indicated at the bottom of the gel.

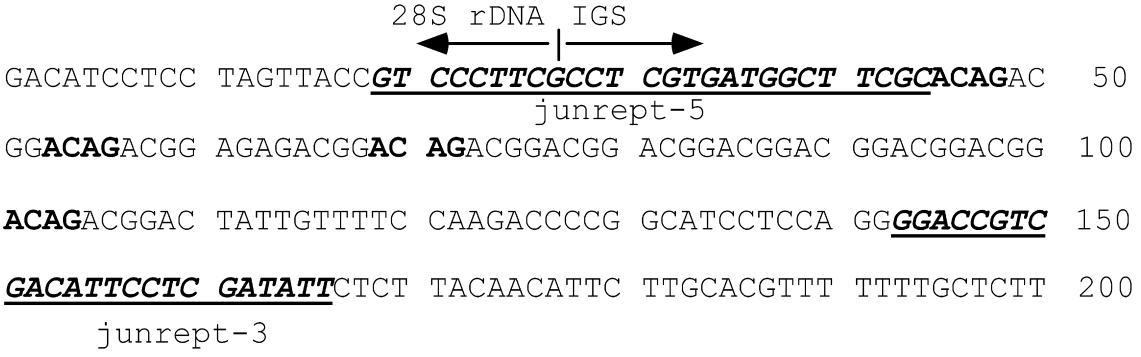

Fig. 1. Primer design for the PCR of IGS-simple sequence repeats. Specific primers (junrept-5 and junrept-3) designed for amplification of tetranucleotide repeats of the IGS are indicated by bold italic letters and underlining; tetranucleotide repeats are indicated by shaded letters.

Among the 30 colonies from Lanyu, only 2 distinct genotypes (A and B) were identified. There are no common length variants shared by these 2 genotypes. Independent samples from the same colony were tested, and identical genotype consistently appeared for each *J. fragilis*, suggesting that IGS-SSR genotyping is repeatable despite it being a multi-copy marker. Twenty individuals (66.67%) represented a single dominant genotype B (Fig. 2). The proportion of distinguishable genotypes, N_c/N, which is frequently used as an index of asexual propagation, was 0.067, whereas genotypic diversity, G_0 was 1.79, and G_0: G_E was 0.597. Genotypic evenness was 0.895 (Table 2). The amount of genetic diversity of the IGS in the weed, *Miscanthus sinensis* var. *glaber* is influenced by the mating system at the organismal level, indicating that the variability documented in the IGS region may reflect the dominant propagation pattern contributing to the local population (Chou et al. 1999). These major deviations from a value of 1 (which would be predicted for freely interbreeding, sexually reproducing populations) in genotypic diversity indices indicate that asexual propagation may play an important role in local population growth of *J. fragilis* at Lanyu. Another alternative explanation for low levels of genetic diversity and low G_0: G_E ratios is a reflection of other population-level processes, other than asexual reproduction, such as inbreeding. Departure from Hardy-Weinberg equilibrium is usually used to elucidate the contribution of asexu-

Table 1. Core sequences corresponding to length variants of the IGS-SSR. Insertions and deletions (indels) are shown by dashes

Variant Genotype	size (bp)	Core sequence of the SSR
A	132	5'-ACAG ACGG AGAG ACGG ACAG $(ACGG)_4$ ACAG $(ACGG)_2$-3'
	164	5'-ACAG ACGG ACAG ACGG AGAG ACGG ACAG ACGG ACAG ACGG ACAG $(ACGG)_7$ ACAG ACGG-3'
	174	5'-ACAG ACGG ACAG ACGG AGAG ACGG --AG ACGG ACAG ACGG $(ACAG)_2$ $(ACGG)_9$ ACAG ACGG-3'
	184	5'-ACAG ACGG ACAG ACGG AGAG ACGG ACAG ACGG ACAG ACGG $(ACAG)_3$ $(ACGG)_{10}$ ACAG ACGG-3'
	188	5'-ACAG ACGG ACAG ACGG AGAG ACGG ACAG ACGG ACAG ACGG $(ACAG)_2$ $(ACGG)_{11}$ ACAG ACGG-3'
B	126	5'-ACAG ACGG AGAG ACGG ACAG $(ACGG)_4$ AC-- ACGG-3'
	128	5'-ACAG ACGG AGAG ACGG ACAG $(ACGG)_4$ ACAG ACGG-3'
	156	5'-ACAG ACGG ACAG ACGG AGAG ACGG ACAG ACGG AGAG ACGG $(ACAG)_2$ $(ACGG)_4$ ACAG ACGG-3'
	160	5'-ACAG ACGG ACAG ACGG AGAG ACGG ACAG ACGG AGAG ACGG ACAG $(ACGG)_5$ ACAG $(ACGG)_2$-3'
	166	5'-ACAG AC-- ACAG ACGG AGAG ACGG ACAG ACGG ACAG ACGG ACAG $(ACGG)_7$ ACAG ACGG-3'
	176	5'-ACAG ACGG ACAG ACGG AGAG ACGG ACAG ACGG ACAG ACGG $(ACAG)_2$ $(ACGG)_9$ ACAG ACGG-3'
	180	5'-ACAG ACGG ACAG ACGG AGAG ACGG ACAG ACGG ACAG ACGG $(ACAG)_2$ $(ACGG)_{10}$ ACAG ACGG-3'

Table 2. Comparison of genotypic diversity measures between *Junceella fragilis* and *Plexaura kuna*

Species/Reef	*N*	N_C	N_C/N	G_0	G_0:G_E	G_0:N_C	Reference
Plexaura kuna							
Macaroon	15	6	0.4	2.92	0.19	0.487	Coffroth and Lasker (1998)
Tiantuopo	37	3	0.07	2.36	0.028	0.393	Coffroth and Lasker (1998)
Sail Rock	20	12	0.08	2.07	0.056	0.69	Coffroth and Lasker (1998)
Junceella fragilis							
Lanyu	30	2	0.067	1.79	0.597	0.895	Present study

al reproduction in a population, however its value is limited in the present study due to the nature of the IGS-SSR used.

PCR IGS-SSR analyses of colonies of the seawhip *J. fragilis* have confirmed the validity of using simple sequence repeats (microsatellites) to detect polymorphisms between individual gorgonian colonies. This technique provides a higher efficiency than do PCR-RFLP and sequence analysis of the complete IGS region in population comparisons of *J. fragilis* (Yang 1996). Although IGS RFLP or sequence variation was successfully applied in revealing patterns of descent in clonal lineages, population genetics, and molecular biogeography of several organisms (Fukunaga et al. 1997. Ganley and Scott 1998, Koch et al. 1998, Barker et al. 1999, Carbone et al. 1999), PCR-RFLP analyses of the IGS of *J. fragilis* using 30 restriction endonucleases failed to identify differences within local populations, and only 2 classic RFLPs were detected between geographically distant populations, while none was found within local populations (Yang 1996, Chen et al. unpubl. data). IGS length variation is generally considered to reflect differences in the number of IGS subrepeat units resulting from unequal crossing-over (reviewed in Gerbi 1985). Further elucidation of the IGS DNA sequences in *J. fragilis* (Chen et al. 2000a) confirmed that length variation detected in the present case (4 bp) was much shorter than any IGS subrepeat unit reported in other species (*Xenopus*: 60 and 80 bp [Moss et al. 1980], *Drosophila*: 90 to 300 bp [Tautz et al. 1987], mouse: 132 bp [Arnheim et al. 1982], and wheat: 136 bp [Baker et al. 1988]), where polymorphisms were detected by RFLP analysis.

PCR IGS-SSR analysis has several advantages over minisatellite DNA fingerprinting, although IGS-SSR strictly represents neither a single- nor a multi-locus method. First, the use of specific primers designed from the IGS sequence avoids the problems of DNA contamination from symbiotic algae (zooxanthellae) and other organisms. *Junceella fragilis* hosts symbiotic algae of clade B (sensu Rowan and Powers 1992), which was demonstrated using *Symbiodinium*-specific primers of small-subunit rDNA in a previous investigation (Smith et al. 1997). Shared bands can usually be found between gorgonian DNA and zooxanthellae DNA in minisatellite DNA fingerprints, which complicates the scoring and interpretation of the autoradiographs (Coffroth et al. 1992). Second, direct genetic assays using the PCR technique require only a small amount of genomic DNA (e.g., 50 ng in the present study), whereas minisatellite DNA fingerprinting needs much larger amounts of high-molecular-weight DNA because of the requirement for RE digestion (e. g. 2 μg of *Plexaura kuna* [Coffroth and Lasker 1998]). Third, PCR IGS-SSR analysis makes the field-based preservation of DNA (using ethanol or DMSO) feasible, increasing the possibility of studying populations from remote regions and facilitating international specimen exchange between countries.

We sampled the seawhips across a reefal area which was about 75 m long. The preliminary statistical data (low G_0, G_0: G_E and, N_C/N) are comparable to values for *Plexaura kuna* at some sites in the Caribbean (e.g., Tiantuopo, Table 2), where clonal propagation is dominant (Coffroth and Lasker 1998). This comparison supports the scenario that success of *J. fragilis* at Lanyu may also be due to vegetative propagation. *Junceella fragilis* tends to occur on reefs where water currents are strong (Chen and Chang 1991). At Lanyu, *J. fragilis* was only found at the reef edge (30 m in depth) at 1 diving site where water currents are strong. Strong currents may help roll away fragments broken from tips of *J. fragilis*, which allows it to dominate the substrate in a short period of time. However, *J. fragilis* still displays a yearly or multi-year cycle of gametogenesis, and spawning of gametes is likely to occur in late August in southern Taiwan (Vermeire 1994). The presence of 2 distinct genotypes sharing no common IGS length variants indicates that these 2 genotypes may have played the role of founders in structuring the local Lanyu population. Application of IGS-SSR analysis to geographically distant populations will further elucidate the clonal diversity of *J. fragilis* and its population structure on a global scale.

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Zoological Studies **41**(3): 295-302 (2002)

以核醣體基因區間之簡單重複序列探討蘭嶼產白蘆莖鞭珊瑚之群體族群遺傳結構

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無性生殖可使同一基因型的群體迅速占據空間，在花蟲動物的生態與進化上扮演著相當重要的角色。而欲進一步闡明群體性對區域族群結構的貢獻需要使用有效的遺傳標誌。我們設計一組增幅位於珊瑚核醣體基因區間簡單重複序列(又稱微衛星)A(C/G)(A/C)G的專一引子，並利用這組引子調查30株產自蘭嶼之白蘆莖鞭珊瑚的群體結構。放射線電泳膠片的分析結果可判斷出12條長度變異的片段，並組合出兩種不同的基因型。其中一種主要的基因型佔66.67%。數個偏低的遺傳歧異度指數（包括 G_0, G_0:G_E,和 N_C/N）支持蘭嶼白蘆莖鞭珊瑚的族群主要以無性生殖，而這兩種基因型可能為蘭嶼海域白蘆莖鞭珊瑚族群的奠基者。本研究證明了應用核醣體基因區間簡單重複序列解析白蘆莖鞭珊瑚在不同尺度族群解構的可行性。

關鍵詞：微衛星，白蘆莖鞭珊瑚，基因型歧異度，核醣體基因區間簡單重複序列，無性生殖。

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