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Polymorphism in soft coral larvae revealed by amplified fragment-length polymorphism (AFLP) markers

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Abstract The dioecious Red Sea soft coral *Parerythropodium fulvum fulvum* breeds its nonsymbiotic planula larvae on the surface of female colonies for less than a week. After completing their development, larvae crawl and settle near maternal colonies. Here we study the genetic polymorphism of developing larvae by the use of amplified fragment-length polymorphism markers. Four reproductive colonies from shallow water populations (two from a dense population and two from a less densely populated area 100 m away) were chosen, and ten larvae were randomly collected from each colony. DNA was analyzed by using three different primer combinations producing 61, 63, 63 polymorphic markers, respectively. All larvae exhibited different banding patterns from one another, illustrating the prominent role of sexual reproduction for the production of larvae. Nei's mean genetic distances for all 12 possible pair-wise combinations for larval origins revealed, in most cases, that sister larvae are genetically closer than larvae from different colonies and that larvae may be grouped into three statistical clusters in accordance with colony origin and population studied. The usefulness of molecular methodologies in coral population genetics is discussed.

Introduction

Population genetic structures of sessile marine invertebrates may be affected by modes of asexual replication, type of mating, larval dispersal potentiality and by settlement and substratum exploitation of larvae (Knowlton and Jackson 1993). For example, limited dispersal of gametes or larvae with the ability to reproduce clonally can give rise to genetic subdivisions as a result of isolation-by-distance (Jackson 1986; Knowlton and Jackson 1993; McFadden and Aydin 1996). These, and other studies revealed that incorrect interpretations may result in cases where scientists are not fully versed on population genetic portraits of sessile marine invertebrates, even on well-studied species. This problem may also arise in cases where methodologies are not well established.

One interesting case study is the Red Sea encrusting soft coral *Parerythropodium fulvum fulvum* (Benayahu and Loya 1983, 1984). This coral, commonly found between 3 and 40 m depth in the reefs of Eilat, is a dioecious species. However, more females than males are found in the shallow water populations (3 to 5 m), whereas the deep reef zone populations (27 to 30 m) are characterized by a 1:1 sex ratio. These differences were assumed to be the outcome of asexual reproductive activities characteristic of shallow water populations (Benayahu and Loya 1983). Colonies of this species are equipped with a gastrovascular system that actively transports symbiont and coral cells (Gateño et al. 1998) and by self–nonself recognition capabilities (Frank et al. 1996). It is an oviparous surface brooder species. Fertilization presumably takes place inside the polyp cavities, followed by spawning that occurs from the end of June till the beginning of August, at dusk, a few days after the new moon and a few days preceding the last quarter (Benayahu and Loya 1983). Embryogenesis takes place on the surface of female colonies within a mucoid suspension. The nonsymbiotic larvae complete their development within 6 d after fertilization and tend to settle immediately upon leaving the maternal colonies. Larvae crawl and adhere to the substratum, forming an aggregated pattern of settlement (Benayahu and Loya 1983).

The mode of surface brooding in larvae is, therefore, an important feature for the spatial pattern of *Parer-*

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ythropodium fulvum fulvum which should be further explored. Although sexual reproduction is presumably the method for juvenile production in this species (Benayahu and Loya 1983), ameiotic (asexual) reproduction in *P. f. fulvum* has not yet been convincingly excluded, especially since a variety of sea anemones (Black and Johnson 1979; Ayre 1984; Edmands 1995; Edmands and Potts 1997; and literature therein) and hard corals (Stoddart 1983; Ayre and Resing 1986; Ayre et al. 1997) are known to produce asexually derived planulae. Moreover, most of the *P. f. fulvum* colonies in the shallow waters of Eilat are females (Benayahu and Loya 1983). This phenomenon, coupled with the sessile mode of life of this species, may favor the system of producing both sexual and asexual planulae, where the production of new juveniles is retained even in the absence of male colonies.

The present study describes the first step in the analyses of the genetic population structure and polymorphism of *Parerythropodium fulvum fulvum* larvae, by using amplified fragment-length polymorphism (AFLP) markers (Vos et al. 1995). One of the main goals is to determine whether larvae of this species are also produced asexually. This novel DNA fingerprinting technique is based on selective PCR amplification of restriction fragments from a total digest of any genomic DNA. Typically, 50 to 100 restriction fragments are amplified and detected on denaturing polyacrylamide gels, without any prior knowledge of nucleotide sequences.

Materials and methods

Sample collection

Parerythropodium fulvum fulvum embryos were collected from the surface of four maternal colonies (Colonies A, B, C and D) at depths between 4 and 6 m, in front of the H. Steinitz Marine Biology Laboratory at Eilat, Red Sea. Colonies A and B were chosen from a dense population (0.5 m from each other). Colonies C and D were chosen from a less dense population (15 m from each other), with no other colonies between them. Both were situated 100 m south of Colonies A and B. Several hundreds of brooded embryos were collected from each one of the four mother colonies by using Pasteur pipettes. They were washed from their mucous sheets with natural filtered seawater and transferred to the laboratory at the National Institute of Oceanography in Haifa in plastic boxes (0.4 liter volume) with filtered seawater. The embryos were kept in plastic boxes with sterile seawater (0.22 μm filtered) at 25 °C, and water was changed every 48 h. Within 5 d, all embryos developed into mature larvae. Ten larvae from each colony were randomly chosen for AFLP analysis.

DNA extraction

The preparation of genomic DNA was in accordance with the protocols of Graham (1978) and ten Lohuis et al. (1990). Larvae were lysed with 100 μl lysis buffer (1 *M* trisborate pH 8.2, 0.5 *M* EDTA, 10% SDS, 5 *M* NaCl) and homogenized gently. Then, 20 μl of NaCl and 120 μl of phenol were added, the solution was mixed and the aqueous phase was collected after 1 min of centrifugation. The aqueous phase was extracted twice with phenol chloroform isoamyl alcohol (25:24:1 v/v/v). Genomic DNA was precipitated with absolute alcohol, washed twice with 70% alcohol, dried and dissolved in sterile water. DNA quality was checked on 0.8% agarose TAE gels.

Preparation of DNA template and AFLP reactions

AFLP analysis was performed using AFLP Analysis System 1 (GibcoBRL, Life Technologies, Paisley, UK) based on the protocol described by Vos et al. (1995). *Mse*I was used as the "frequent" cutter and *Eco*RI as the "rare" cutter. Restriction enzyme digests were performed in a total volume of 25 μl for 2 h at 37 °C. Following heat inactivation of the restriction endonucleases, the genomic DNA fragments were ligated to the *Eco*RI adapter 5′-CTCGTAGACTGCGTACC, CTGACGCATGGTTAA-5′ and to the *Mse*I adapter 5′-GACGATGAGTCCTGAG, TACTCAGGACTCAT-5′ to generate template DNA for amplification (http://carnegiedpb.stanford.edu/methods/aflp.html). PCR was performed in two consecutive reactions: in the preamplification step, genomic DNA fragments were amplified with two primers: an *Eco*RI Oligo primer (5′-CTCGTAGACTGCGTACC-3′) and a *Mse*I Oligo primer (5′-GACGATGAGTCCTGAG-3′). The PCR products of the preamplification reaction were diluted and used as a template for the selective amplification using three combinations of AFLP primers each containing three different nucleotides (*Eco*RI primer: 5′-AGACTGCGTACCAATTCACA-3′ and *Mse*I primer: 5′-GATGAGTCCTGAGTAACAT-3′; E-AGG and M-CAG; E-ACG and M-CTG. E and M refer to additional selective nucleotides at the 3′-end of the primer). The *Eco*RI selective primer was ^{33}P-labeled before amplification. Products from the selective amplification were separated on a 7.5% denaturing polyacrylamide (sequencing) gel. The resultant banding pattern ("fingerprint") was visualized by autoradiography and analyzed manually. Nei's genetic distance (1972, 1978) was calculated using the Tools for Population Genetic Analyses (TFPGA) Program, Version 1.3 (M.P. Miller 1997; http://herb.bio.nau.edu/~miller/tfpga.htm).

Results

A total of 39 aposymbiontic larvae from four mother colonies were analyzed (DNA of one larva from Colony A was damaged and excluded from the experiment). Amplified fragment sizes were 100 to 400 bp (Fig. 1). Several of the larvae analyzed in each primer combination (Fig. 1; Table 1) resulted in unclear band patterns and were excluded from the specific set of analyses. Depending on the primer combination (E-ACA, M-CAT; E-AGG, M-CAG; E-ACG, M-CTG) totals of 61, 63, 63 bands (markers), respectively, were analyzed. Most of the bands (55.7% to 88.9%, Table 1) were polymorphic. All genetic distances were calculated by using only the polymorphic markers. With regard to the fingerprint profiles, all 39 larvae exhibited different banding patterns from one another. Only two sister larvae from Colony A presented identical band patterns under a single primer combination (E-ACA, M-CAT), but they differed in the other two primer combinations. All 39 larvae therefore were genetically distinct from each other.

Mean genetic distances (Nei 1972, 1978) for all 12 possible combinations of larva origin [among sister larvae (A-A, B-B...) versus larvae of different origins (A-B, A-C....)], calculated for each primer combination are

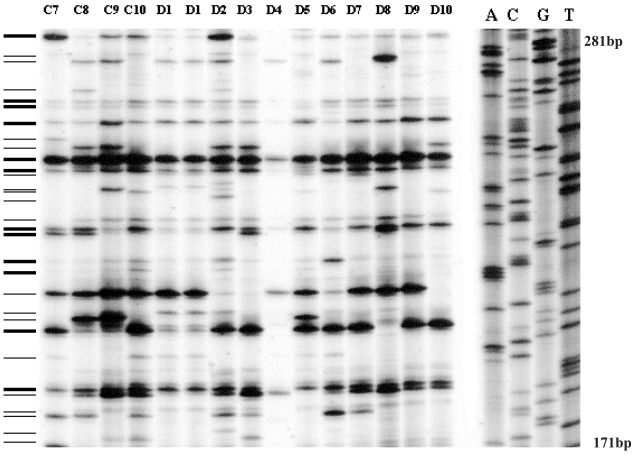

Fig. 1 *Parerythropodium fulvum fulvum*. Part of a representative AFLP (amplified fragment-length polymorphism) profile carried out on 14 larvae. Each larva is designated by a capital letter (C, D) for the mother origin, followed by a number. Two replicate samples were taken from larva D1 depicting the reproducibility of the band pattern. Scored bands are marked on the left. Larva D4 reproduced weak bands and was deleted from the analysis. The primer combination E-AGG and M-CAG was used. DNA fragment sizes ranged from 100 to 400 bp. A, C, G, T (right columns) are the nucleotide sequences

Table 1 *Parerythropodium fulvum fulvum*. Summary of larvae tested, number of AFLP bands scored and markers used for each of the primer combinations employed

	Primer combination		
	E-ACA, M-CAT	E-AGG, M-CAG	E-ACG, M-CTG
No. of larvae, Colony A	6	8	9
No. of larvae, Colony B	8	8	9
No. of larvae, Colony C	8	10	9
No. of larvae, Colony D	6	9	10
No. of bands scored	61	63	63
No. of polymorphic bands	34 (55.7%)	56 (88.9%)	56 (88.9%)

shown in Table 2. The values between sister larvae were usually (34 out of 36 cases; except in combinations B-B versus B-C and B-B versus B-D for one primer combination) smaller than those between unrelated larvae. In most combinations (7/12 to 10/12, depending on the primer combination; Table 2), the mean genetic distances among sister larvae were significantly lower than those calculated for between-colony larvae. These results are consistent for all primer combinations as shown by the low coefficient of variability among the three calculated mean genetic distances (Table 3). When analyzing within-group variations, the genetic distances among sister larvae varied with colony origin. Larvae from Colony C showed the lowest values, followed by Colonies D, A and B (Table 3). Moreover, the mean genetic distances of the four colonies' larvae may be grouped into three statistical clusters (ANOVA, Duncan's multiple range test; $p < 0.05$) in which the genetic distances of Colonies A and B are not significantly different from each other but differ from Colonies C and D which are not grouped together. The MGD average values, grouped for the dense population (A-A, A-B, B-B), are unexpectedly higher than those calculated for the less dense population (C-C, C-D, D-D; 0.347 vs 0.257, respectively).

Discussion

Our results reveal extensive polymorphism among larvae from the same mother colony (each one of the primer combinations used revealed 61 to 63 scored bands of which 55.7% to 88.9% were polymorphic). Band analyses revealed that all 39 larvae studied were genetically distinct from each other, indicating only sexual production of larvae in the gonochoric surface brooder soft coral *Parerythropodium fulvum fulvum*, as suggested by

Table 2 Mean genetic distance (Nei 1972, 1978) analyses between larvae from the same mother colony (sister larvae) versus larvae from different mother colonies (unrelated larvae). Duncan grouping outcomes: * = $p < 0.05$; NS = not significant. ANOVA values are: $df = 368$, $F = 12.84$, $p < 0.001$; $df = 551$, $F = 21.3$, $p < 0.001$; $df = 656$, $F = 24.29$, $p < 0.001$, respectively, for each primer combination

Analysis combination	Primer combination					
	E-ACA, M-CAT		E-AGG, M-CAG		E-ACG, M-CTG	
	Mean genetic distances	Significance	Mean genetic distances	Significance	Mean genetic distances	Significance
A-A vs A-B	0.302 vs 0.404	*	0.302 vs 0.332	NS	0.359 vs 0.443	*
A-A vs A-C	0.302 vs 0.344	NS	0.302 vs 0.360	*	0.359 vs 0.410	*
A-A vs A-D	0.302 vs 0.395	*	0.302 vs 0.417	*	0.359 vs 0.420	*
B-B vs A-B	0.364 vs 0.404	NS	0.313 vs 0.332	NS	0.294 vs 0.443	*
B-B vs B-C	0.364 vs 0.344	NS	0.313 vs 0.362	*	0.294 vs 0.311	NS
B-B vs B-D	0.364 vs 0.358	NS	0.313 vs 0.369	*	0.294 vs 0.335	NS
C-C vs A-C	0.198 vs 0.344	*	0.232 vs 0.360	*	0.194 vs 0.410	*
C-C vs B-C	0.198 vs 0.344	*	0.232 vs 0.362	*	0.194 vs 0.311	*
C-C vs C-D	0.198 vs 0.275	*	0.232 vs 0.278	*	0.194 vs 0.343	*
D-D vs A-D	0.265 vs 0.395	*	0.277 vs 0.417	*	0.247 vs 0.420	*
D-D vs B-D	0.265 vs 0.358	*	0.277 vs 0.369	*	0.247 vs 0.335	*
D-D vs C-D	0.265 vs 0.275	NS	0.277 vs 0.278	NS	0.247 vs 0.343	*

Table 3 Average of the mean genetic distances (MGDs ± SD) and the coefficient of variability (CV = 100 SD/mean) between sister larvae for the three primer combinations

Colonies	Average MGDs ± SD	CV (%)
A-A	0.321 ± 0.033	10.2
B-B	0.323 ± 0.036	11.2
C-C	0.208 ± 0.021	10.0
D-D	0.263 ± 0.015	5.6
A-B	0.393 ± 0.056	14.3
A-C	0.371 ± 0.034	9.3
A-D	0.410 ± 0.014	3.3
B-C	0.339 ± 0.026	7.6
B-D	0.354 ± 0.017	4.9
C-D	0.299 ± 0.038	12.9

Benayahu and Loya (1983). Furthermore, the mean genetic distances among sister larvae are, as expected, lower than the genetic distances among larvae from different maternal colonies. The differences recorded among sister larvae are probably the outcome of the genetic distances between the parents (the mother colonies and sperm donors) and may also be affected by the number of males fertilizing a specific female colony. This is probably the reason why the combined average of MGDs in the dense population is unexpectedly higher than in the less dense population. More sperm donor colonies will raise the polymorphism of the produced larvae. Larvae of Colonies A and B, which are about 0.5 m from each other and which are located within a dense *P. f. fulvum* population, have similar, significantly higher mean genetic distances (which again reflected fertilization by more than a single sperm donor). Colonies C and D were chosen from a less crowded population (15 m from each other without any other *P. f. fulvum* colony between them) about 100 m further south of the dense population. Larvae originating from Colonies C and D exhibited significantly lower mean genetic distances as compared to larvae from Colonies A and B, which, in addition, are significantly different from each other. Together these results, coupled with the low coefficient of variability recorded between the three pairs of primer combinations, further emphasize the efficiency of using AFLP.

Any population genetic analysis requires the accurate identification of individual genotypes. AFLP is one of the best recently developed genetic techniques for identifying variations with high resolution at the level of the individual's DNA (Parker et al. 1998). In the phylum Cnidaria, several other techniques have already been employed such as DNA fingerprints (Coffroth et al. 1992; Coffroth 1997; Edmands and Potts 1997), randomly amplified polymorphic DNA (RAPDs; Coffroth and Mulawka 1995; Lasker et al. 1996; Brazeau et al. 1998) and even AFLP on a scleractinian coral (Lopez and Knowlton 1997). Unfortunately, we could not use this or either one of the above techniques to screen the maternal colonies or other nearly settled *Parerythropodium fulvum fulvum* colonies. The symbiotic zooxanthellar DNA may interact and add additional uncontrolled factors. The larvae of this species which lack symbiotic algae provide, on the other hand, suitable material for such molecular approaches.

It is well documented that among benthic marine invertebrates the dispersal of larvae constitutes the primary source of continuing gene flow (Knowlton and Jackson 1993; McFadden and Aydin 1996; Yu et al. 1999). Broad dispersal of propagules should genetically homogenize populations. In contrast, geographically restricted gene flow enhances differentiation among populations (Yu et al. 1999). Recruitment from mother colonies or from nearby populations is not the only factor that shapes the genetic population structures of *Parerythropodium fulvum fulvum* in Eilat. Random selection acting on patches of larvae (Burnett et al. 1994), brooding of larvae and the limited dispersal of the sexually produced propagates (Benayahu and Loya 1983, 1984), clonal propagation (Barki unpublished data) and the skewed sex ratios (Benayahu and Loya 1983) which may potentially reduce the numbers of sperm donors, all probably contribute to the small-scale patchiness in the genetic structure of shallow water *P. f. fulvum* populations in Eilat. Genetic differentiation within a subpopulation can therefore be expected. Further work on the genetic structure within and between populations is therefore needed to establish this prediction.

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References

Ayre DJ (1984) The effects of sexual and asexual reproduction on geographic variation in the sea anemone *Actinia tenebrosa*. Oecologia 62: 222–229

Ayre DJ, Hughes TP, Standish RJ (1997) Genetic differentiation, reproductive mode, and gene flow in the brooding coral *Pocillopora damicornis* along the Great Barrier Reef, Australia. Mar Ecol Prog Ser 159: 175–187

Ayre DJ, Resing JM (1986) Sexual and asexual production of larvae in reef corals. Mar Biol 90: 187–190

Benayahu Y, Loya Y (1983) Surface brooding in the Red Sea soft coral *Parerythropodium fulvum fulvum* (Forskål). Biol Bull mar biol Lab, Woods Hole 165: 353–369

Benayahu Y, Loya Y (1984) Substratum preferences and planulae settling of two Red Sea alcyonaceans: *Xenia macrospiculata* Gohar and *Parerythropodium fulvum fulvum* (Forskål) J exp mar Biol Ecol 83: 249–261

Black R, Johnson MS (1979) Asexual viviparity and population genetics of *Actinia tenebrosa*. Mar Biol 53: 27–31

Brazeau DA, Gleason DF, Morgan ME (1998) Self-fertilization in brooding hermaphroditic Caribbean corals: evidence from molecular markers. J exp mar Biol Ecol 231: 225–238

Burnett WJ, Benzie JAH, Beardmore JA, Ryland JS (1994) High genetic variability and patchiness in a common Great Barrier Reef zoanthid (*Palythoa caesia*). Mar Biol 121: 153–160

Coffroth MA (1997) Molecular approaches to the study of clonal organisms: deciphering the alphabet soup. In: Lessios HA, MacIntyre IG (eds) Proc 8th Int Coral Reef Symp. Vol. 2.

Smithsonian Tropical Research Institute, Balboa, Panama, pp 1603–1608
Coffroth MA, Lasker HR, Diamond ME, Bruenn JA, Berminhgam E (1992) DNA fingerprints of a gorgonian coral: a method for detecting clonal structure in a vegetative species. Mar Biol 114: 317–325
Coffroth MA, Mulawka JM (1995) Identification of marine invertebrate larvae by means of PCR-RAPD species-specific markers. Limnol Oceanogr 40: 181–189
Edmands S (1995) Mating systems in the sea anemone genus *Epiactis*. Mar Biol 123: 723–733
Edmands S, Potts DC (1997) Population genetic structure in brooding sea anemones (*Epiactis* spp.) with contrasting reproductive modes. Mar Biol 127: 485–498
Frank U, Bak RPM, Rinkevich B (1996) Allorecognition responses in the soft coral *Parerythropodium fulvum fulvum* from the Red Sea. J exp mar Biol Ecol 197: 191–201
Gateño D, Israel A, Barki Y, Rinkevich B (1998) Gastrovascular circulation in an octocoral: evidence of significant transport of coral and symbiont cells. Biol Bull mar biol Lab, Woods Hole 194: 178–186
Graham DE (1978) The isolation of high molecular weight DNA from whole organisms or large tissue masses. Analyt Biochem 85: 609–613
Jackson JBC (1986) Modes of dispersal of clonal benthic invertebrates: consequences for species distributions and genetic structure of local populations. Bull mar Sci 39: 588–606
Knowlton N, Jackson JBC (1993) Inbreeding and outbreeding in marine invertebrates. In: Thornhill NW (ed) The natural history of inbreeding and outbreeding. University of Chicago Press, Chicago, pp 200–249
Lasker HR, Kim K, Coffroth MA (1996) Reproductive and genetic variation among Caribbean gorgonians: the differentiation of *Plexaura kuna*, new species. Bull mar Sci 58: 277–288
Lopez JV, Knowlton N (1997) Discrimination of species in the *Montastraea annularis* complex using multiple genetic loci. In: Lessios HA, MacIntyre IG (eds) Proc 8th Int Coral Reef Symp. Vol. 2. Smithsonian Tropical Research Institute, Balboa, Panama, pp 1613–1618
McFadden CS, Aydin KY (1996) Spatial autocorrelation analysis of small-scale genetic structure in a clonal soft coral with limited larval dispersal. Mar Biol 126: 215–224
Nei M (1972) Genetic distance between population. Am Nat 106: 283–292
Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583–590
Parker PG, Snow AA, Schug MD, Booton GC, Fuerst PA (1998) What molecules can tell us about populations: choosing and using a molecular marker. Ecology 79: 361–382
Stoddart JA (1983) Asexual production of larvae in the coral *Pocillopora damicornis*. Mar Biol 76: 279–284
ten Lohuis M, Alderslade P, Miller DJ (1990) Isolation and cloning of DNA from somatic tissue of soft corals (Cnidaria: Octocorallia). Mar Biol 104: 489–492
Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407–4414
Yu JK, Wang HY, Lee SC, Dai CF (1999) Genetic structure of a scleractinian coral, *Mycedium elephantotas*, in Taiwan. Mar Biol 133: 21–28