Development and Application of Genetic Markers for Population Structure Analysis of the Blue Coral Reef Starfish, *Linckia laevigata* (Linn.) (Echinodermata: Asteroidea)

Richard M. Magsino*, Marie Antonette R. Juinio-Meñez, and **Rachel V. Ravago**
Marine Science Institute, College of Science
University of the Philippines, Diliman, Quezon City 1101 Philippines
Tel. No.: (632) 920-5301 local 7430; TelFax: (632) 924-7678; E-mail: rickym@upmsi.ph

ABSTRACT

The tropical blue coral reef starfish, *Linckia laevigata,* is a good model species for examining genetic affinities among reef populations. Allozyme and mtDNA PCR-RFLP genetic markers were developed for this species. A total of nine (9) polymorphic and three (3) monomorphic allozyme marker loci were resolved out of 25 enzyme systems assessed for genetic activity in three electrophoretic buffers used. Polymorphic mitochondrial DNA gene segments of the control region with flanking sequences and the cytochrome oxidase I (CO1) were amplified after examining several gene regions for PCR product amplifications. Restriction enzyme screening of the CO1 region revealed variation of restriction profiles in seven (7) out of twenty (20) enzymes initially tested. Preliminary comparison of the genetic structure of *L. laevigata* based on allozyme and mtDNA markers for selected reefs are presented. The development of these genetic markers will be useful in inferring gene flow and reef connectivity in the South China Sea, Palawan shelf, and Sulu Sea.

Keywords: genetic markers, allozymes, PCR-RFLP, *Linckia laevigata*, population genetics, Asteroidea

INTRODUCTION

The development of genetic markers is an essential step towards the proper evaluation of genetic relatedness within and between populations. Allozymes and mtDNA restriction fragment length polymorphisms are techniques used in assaying protein and DNA-level variation, respectively. Allozymes are well established as markers in evaluating genetic variation among populations. The application of this technique to genetic studies of geographically separated populations of marine invertebrates has significantly increased our understanding of population differentiation in the marine environment and the levels and patterns of connectivity between reef populations (Richardson and others 1986). On the other hand, investigation of mitochondrial DNA genetic variation through polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) technique provides a potentially more powerful means of establishing genetic affinities between reef populations, particularly in cases where allozyme polymorphism is limited. While allozyme analysis is based on variations in gene products (i.e., proteins), DNA analysis is based on actual variation in the DNA sequence. The more extensive genetic divergence in mtDNA results in greater variability in the RFLPs compared to nuclear data, hence, enhanced resolving power in detecting genetic variability (Ovenden 1990).

* *Corresponding author*

Concordance of population genetic structure based on protein and DNA-level markers will strengthen inferences on gene flow among reef systems.

The tropical blue coral reef starfish, *Linckia laevigata* (Linn.), has a long larval life; it remains in the water column up to 28 days before settling. This suggests potential dispersal across large geographic distances (Yamaguchi 1977). The species has a wide geographic distribution in the Pacific and Indian oceans, frequently in shallow reef systems. The species is renowned for its ubiquitous distribution, occurring in different color morphs, such as royal blue and salmon-orange, although some intermediate colors also occur (Williams and Benzie 1998).

This paper reports the results of the development of allozyme and DNA-level genetic markers for *L. laevigata*. The markers were initially applied to a subset of populations obtained from study sites in the Sulu Sea, the Palawan shelves, and the South China Sea. Genetic markers were examined to investigate whether results on patterns in genetic variation will be concordant for both markers as part of the assessment of the utility of these markers for population genetics studies.

MATERIALS AND METHODS

Sample collection

Collection of *L. laevigata* was done by SCUBA and snorkeling across selected shallow reef areas in the Sulu Sea and the South China Sea (Fig. 1). The pyloric caeca of individual starfish was obtained by making an incision across each arm with a hacksaw. Tissue samples were rinsed with seawater, blotted with clean tissue paper, and then placed inside small, appropriately labeled zip-lock plastic bags. Tissues were frozen immediately in liquid nitrogen, transported in dry ice, and stored at -70°C until use (Williams and Benzie 1993).

Allozyme-level marker development

Protein extraction. Optimized method of protein enzyme extraction was performed following Williams (1992). Frozen chips of pyloric caeca samples were placed individually in a cold microwell plate. An extraction buffer composed of 0.04% mercaptoethanol colored with bromophenol blue was added to the tissue samples homogenized using a glass rod.

Electrophoresis and staining. Electrophoretic procedures followed the protocols of Williams (1992) with a few modifications. A 12% starch gel (Sigma Co.) was prepared using various buffers. Filter paper wicks were soaked in homogenized tissue samples and then applied to the gel. Electrophoresis was performed in a BioRad electrophoresis chamber (BioRad Co.) at 45 mAmps and ~ 350 V for 7-8 hours. Staining of gel slices followed the procedures in Shaw and Prasad (1970). Activity and resolution of enzymes on different buffer systems were evaluated. Allozyme banding patterns were recorded as soon as the bands appeared on the gels as prolonged staining often leads to smearing. Isozymes coded by separate loci were numbered in the order of decreasing anodal mobility. Electromorphs were equated with alleles and coded in the order of decreasing anodal mobility. A value of 100 was assigned to the most common allele at each locus.

DNA-level marker development

DNA extraction. DNA was extracted from *L. laevigata* using the frozen pyloric caeca tissue samples through the Rapid One Step Extraction (ROSE) procedure of Steiner et al. (1995). Excised tissue ~ 25 mg was placed in a sterile, pre-chilled mortar and pestle, and flash-frozen with liquid nitrogen. The tissues were pulverized rapidly; ~10 mg of which was transfered into a 1.5 ml microcentrifuge tube containing 200 µl ROSE buffer. Tubes were then incubated in a 90 °C waterbath for 20 min with frequent shaking, chilled in an ice bath for 5 min, and centrifuged for 2-3 min at 15000 rpm. The supernatant was transfered into new 0.5 ml microcentrifuge tubes. Crude DNA extracts were run on 0.08% agarose gels and stained with ethidium bromide for visualization. DNA was quantified using standard spectrophotometric protocols.

Polymerase chain reaction-restriction fragment length polymorphism. Optimization of PCR conditions using different primer pairs were conducted. These

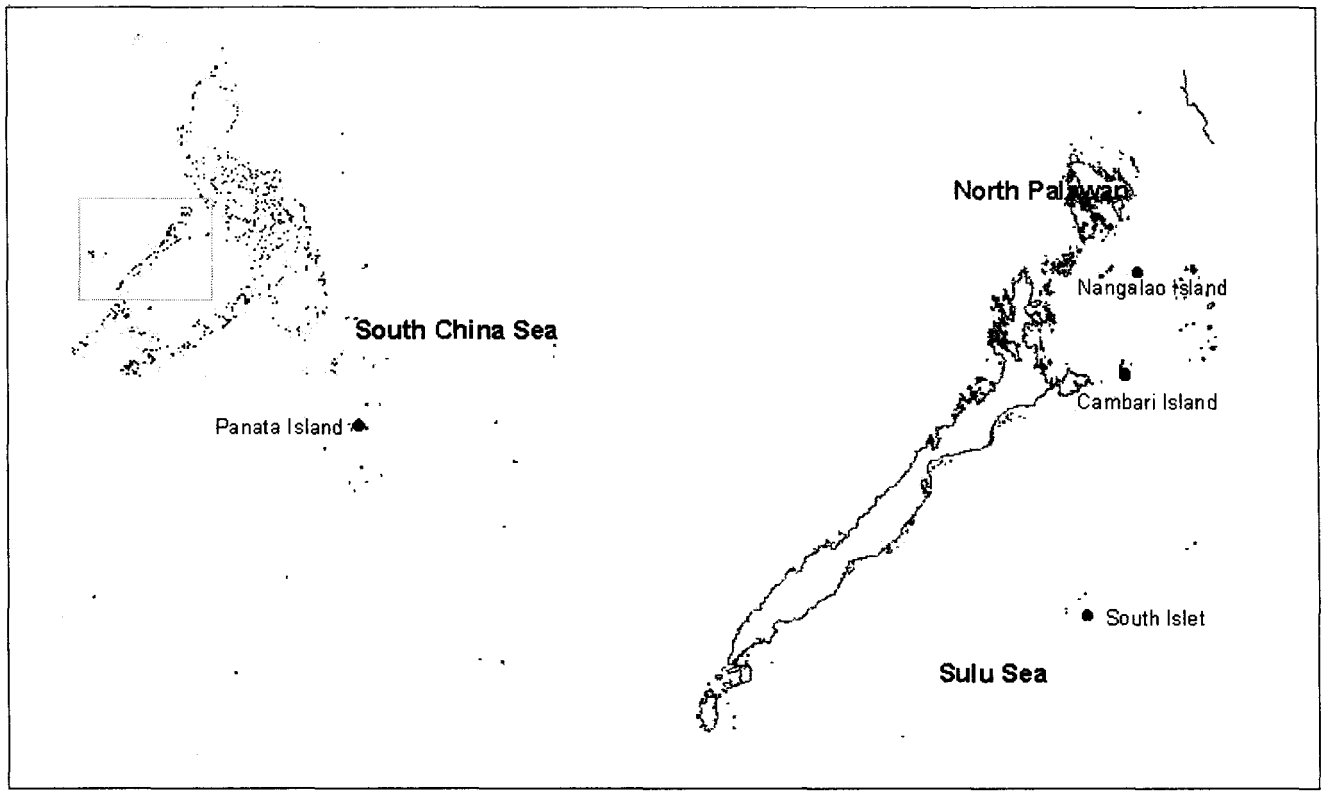

Fig. 1. Collection sites of *Linckia laevigata* in the Sulu Sea and South China Sea

involved optimizing concentrations of $MgCl_2$, DNA templates, Taq polymerase DNA, annealing temparatures and time, and number of cycles. PCR cycling parameters were performed in 10 µl-capillary tubes in an Air Thermal Cycler (Idaho Technology). Scale up PCR amplifications for restriction digestion was performed in 50 µl-capillary tubes.

Restriction enzyme digestion (GIBCO-BRL or Pharmacia) of PCR-amplified products was conducted following the manufacturer's instructions. The restriction reaction was prepared by mixing components (distilled water, 10x buffer, 4-5 µl PCR product and 1U restriction enzyme). The reaction was then incubated in a water bath at the appropriate temperature for 1.5-2 h. The reaction was stopped by adding loading buffer and loaded into a 1% agarose gel (GIBCO-BRL). Restriction fragments were separated by electrophoresis at 50-100V for 40-60 min. The KODAK Digital Science 1D Image Analysis Software was used to document and estimate the size of the restriction fragments.

Statistical analyses of genetic data

Analysis of *L. laevigata* allozyme (N=110) and PCR-RFLP (N=26) data were conducted for samples collected from four (4) sites in the Sulu Sea, North Palawan, and South China Sea (e.g., South Tubbataha Reefs, Cambari Island, Nangalao Island and Panata Island).

Allozyme data. BIOSYS-1 (Swofford and Selander 1981) was used to analyze genotypic data obtained from allozyme markers. Calculation of Nei (1978) unbiased genetic distance was performed and a dendrogram based on D was also obtained. Weir & Cockerham (1984) F-statistics (F_{st} and F_{is} values) was conducted using the Tools for Population Genetics Analysis (TFPGA) package of Miller (1991). Significance of F_{st} and F_{is} values was calculated using the equation of Waples (1987).

Mitochondrial DNA PCR-RFLP data. PHYLIP (Phylogenetic Inference Package) (Felseinstein 1993)

was used to calculate gene frequencies and continuous characters maximum likelihood on CONTML program. Genetic distances using data on gene frequencies were then obtained in the GENDIST program based on the equations of Nei (1978). Cluster analysis utilizing UPGMA neighbor joining algorithm was generated using the NEIGBOR program.

RESULTS AND DISCUSSION

Allozyme-level marker development

After screening about twenty five (25) enzyme systems in three (3) electrophoretic buffers: Tris-Citric acid (TC pH 7), Tris-Maleic acid (TM), and Lithium hydroxide (LiOH), a set of 10 enzyme systems corresponding to 12 loci was identified to have good activity on TC pH 7 buffer. Of the 12, nine (9) loci were polymorphic and three (3) were monomorphic (i.e., MDH-1, LGG-1 and LT-1). Six of the polymorphic allozymes were similar to those identified by Williams & Benzie (1993) for the same species: GPI-1, HK-1, SOD-1, LP-1, LGG-1 and LT-1. Three (3) additional polymorphic loci were resolved: isocitrate dehydrogenase (IDHP), mannose-6-phosphate isomerase (MPI), and phosphoglucomutase (PGM); malate dehydrogenase (MDH) was monomorphic.

The number of alleles in each polymorphic loci ranged from 3-5, lower than the reported number of alleles for the same species in the Great Barrier Reefs Australia (Williams and Benzie 1993). However, the values were comparable to the Philippine samples of *L. laevigata* in Williams and Benzie (1996) collected from Bolinao, Pangasinan and Dumaguete, Negros Island. For population genetics studies it is often appropriate to analyze at least six (6) variable marker loci to be able to have reliable genetic data (Richardson et al. 1986) thus, nine (9) polymorphic allozyme markers will provide better estimations of genetic variability of the populations studied.

One (1) zone of activity was observed for GPI, HK, SOD, IDHP, MPI, and LP loci. Two (2) zones of activity were identified for peptidases, LGG and LT, with the faster locus polymorphic. Heterozygotes in GPI-1, SOD-1, IDHP-1, MPI-1, and LP-1 appeared as three-banded dimeric phenotypes while homozygotes appeared as one-banded phenotypes. Two-banded heterozygote phenotype was observed for HK-1, LT-1, and LGG-1 loci, indicating monomeric protein structure (Fig. 2).

SOD-1 locus appeared as white bands on a dark background. Oversmearing of the GPI-1 locus, on the other hand was prevented by backstaining with 6% acetic acid. Special care was taken for the three (3) peptidases as the gels stained dark enough to require almost immediate scoring before they were over-stained. Overall, the resolution of these loci from samples in the SCS and Sulu Sea was good enough to permit easy scoring.

DNA-level marker development

The development of DNA-level genetic markers for *L. laevigata* can provide finer resolution of population differentiation by examining variation in nucleic acid

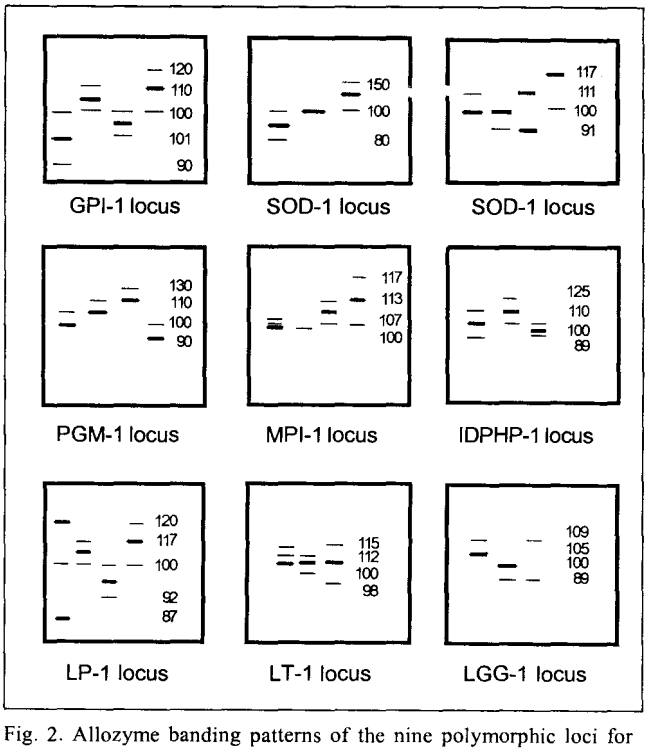

Fig. 2. Allozyme banding patterns of the nine polymorphic loci for *Linckia laevigata*

sequences. Unlike allozymes which are allelic variation in gene products, RFLP markers reflect variation in DNA sequences of particular regions of the mitochondrial DNA. In this study, primers for several mitochondrial DNA regions were initially tested for amplification in *L. laevigata*. These include the control region, 16S ribosomal RNA, 16S rRNA-COI genes, and the ATPase6-cytochrome oxidase III (ATCO) segments. Initial DNA samples were amplified using the control region and 16S primer pairs, but were not replicated in subsequent runs. The 16S rRNA-CO1 and ATCO gene regions were not successfully amplified.

Thus, there was a need to design specific primers for other regions of the genome which are longer and which are potentially polymorphic. An echinoderm-specific primer pair sequence for the COI region (ApCOI) was designed based on the complete mitochondrial gene sequence available for another starfish, *Asterina pectinifera* (Asakawa and others 1993) (NCBI GenBank database). Optimization of PCR conditions yielded no PCR product amplification.

Several studies on phylogenetics and population genetics have focused on the analysis of the mitochondrial DNA control region. In particular, Williams and Benzie (1997) evaluated genetic differentiation of populations of *L. laevigata* in Indo-West Pacific regions using variations derived from the mitochondrial DNA control region with flanking sequences. The D-loop region which is in the control region, accumulates mutations at the fastest rate and is therefore, useful for population-level studies. Thus, the mitochondrial DNA control region fragment was also assessed for the SCS-Sulu Sea samples. The 12SA and 16SB primer pair (Williams & Benzie 1997) (5'-3'ACACATCGCCCGTCACTCTC and GACGAGAAGACCCTATCGAGC) amplified a 1100 bp containing the putative control region, 2 tRNA genes (i.e., tRNA-Glu and tRNA-Thr); the 3'end of the 12S rRNA gene and the 3'end of the 16S rRNA gene segments. However, inconsistency in amplification of the region indicates the need for further optimization.

PCR products of the COI gene segment of *L. laevigata* were consistently amplified using primer pairs of Williams (pers. comm. 1998). Agarose gel electrophoresis of PCR-amplified region did not reveal any variation in product size among individuals. Initial restriction analysis

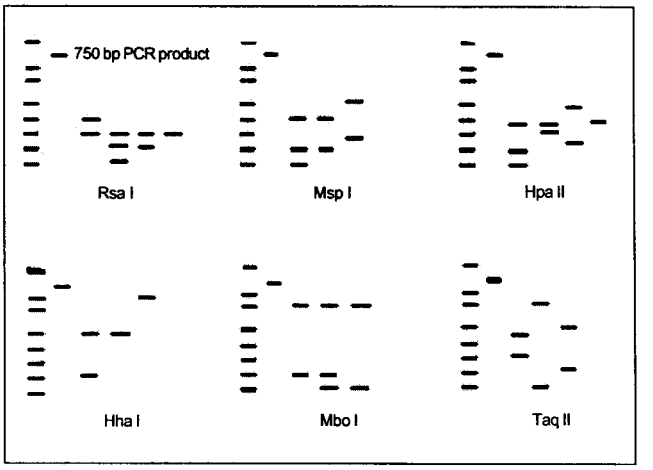

Fig. 3. RFLP haplotypes generated from the COI gene segment of *Linckia laevigata*

of 26 DNA samples of *L. laevigata* from Panata Island in the SCS, Nangalao, and Cambari Islands in the North Palawan shelf and South Tubbataha in the Sulu Sea revealed polymorphism at seven (7) out of 20 restriction enzymes assessed (Fig. 3). Profiles for three (3) enzymes were monomorphic while 10 enzymes did not recognize any cutting site. Polymorphic restriction enzymes assessed identified more than one restriction fragment pattern owing to the loss and/or gain of one or more sites. The number of haplotype variants identified for each enzyme ranged from two (2) to four (4) for all variable loci. Banding patterns were usually unambiguous, except for some bands which were not detected in the gel due to small fragment size (<80 bp).

Comparison of genetic structures

A dendrogram of allozyme genetic affinities between populations based on Nei (1978) unbiased values is presented (Fig. 4a). Three clusters were observed: (1) SCS, North Palawan and Sulu Sea, Panata Island on the SCS and Cambari on North Palawan shelf clustered together; (2) Nangalao Island, also on north Palawan clustered separately; (3) South Islet of Tubbataha Reefs was the most distinct among the populations investigated. Weir and Cockerham (1984) F-statistics measures with 95% confidence interval from bootstrapping over loci, with 1000 replications performed. These gave F_{st} values ranging from 0.0137 to 0.0628 and F_{is} from 0.0147 to 0.2933 with jacknifed mean F_{st} and F_{is} estimates of 0.0326 (significant at p = 0.005) and 0.1186 (ns), respectively. On the other hand,

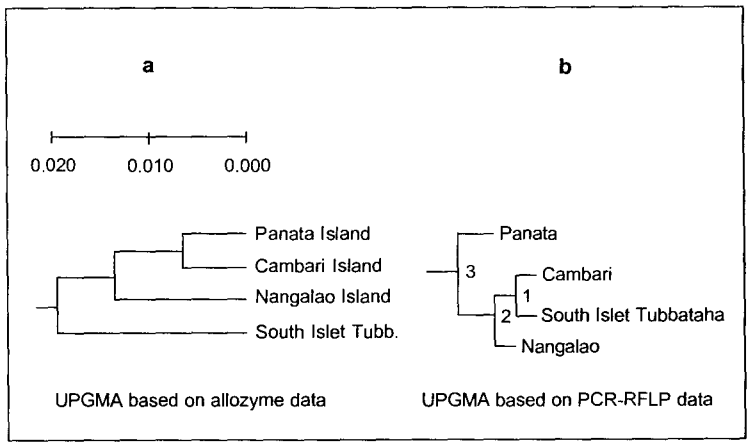

Fig. 4. Dendrograms based on the preliminary comparison of allozyme (a. N = 110) and PCR-RFLP (b. N = 26) data showing genetic relatedness of *Linckia laevigata* from selected sites in the SCS and the Sulu Sea.

the dendrogram based on RFLP haplotype variation (Fig. 4b) revealed three (3) groups. However, the clustering of populations was different from that based on the allozyme data. South Islet Tubbataha samples clustered with Cambari reef populations forming the first cluster. Nangalao reef, which is also located in the north Palawan shelf with Cambari population, was different, forming the second node. Panata reef population on the SCS shoal was the most distinct among the four populations surveyed. Statistical tests to determine significance between groups were conducted for allozyme data, but not for RFLP data due to the small sample size used.

Although allozyme markers have been previously developed for *L. laevigata* (Williams 1992, Williams and Benzie 1993), three (3) new polymorphic markers were identified in this study. These, together with the development of a mt DNA marker from the COI gene region, provide additional tools to assess population level genetic variation of this species. Comparison of population genetics based on allozyme and DNA-level markers will provide additional insights about factors that contribute to population structuring of this reef organism. An important consideration is that DNA level markers are less likely to be subjected to selection compared to allozyme markers (Ward and Grewe 1995).

Analyses of genetic data using DNA-level markers in conjunction with allozymes are limited. Williams and Benzie (1997) assessed the genetic structuring of *L. laevigata* in the IndoWest Pacific using the mitochondrial DNA control region and found significant differentiation among populations, in contrast to allozymes which revealed homogeneous panmictic mixing among reefs. Initial analysis in this study showed discordance on the patterns of genetic clustering of selected populations of *L. laevigata*. The disparity, however, may be attributed to the small sample size used in the DNA analysis (i.e., sample size ranged from 5-10 per population). To better assess the utility of the COI region DNA markers in delineating population structure, more samples need to be analyzed from each site. In addition, investigation of other regions of the genome which are longer and potentially more polymorphic (e.g., the D-loop or the control region) should also be conducted. The development of DNA markers for *L. laevigata*, together with allozymes will be useful in inferring the extent of gene flow among reef populations in the South China Sea and, the Sulu Sea.

ACKNOWLEDGMENTS

We thank Dr. P.M. Aliño (UPMSI) for his supervision of the field collection of samples; S.T. Williams (STRI Panama) for providing DNA primer sequences; and the invertebrate collection team (E. Pacis, H. Roa-Quiaoit, M. Talaue, M. Malay, M. Sison, and T. Hamada) for providing assistance in the collection of samples. This project was funded by the Department of Science and Technology-Philippine Council for Aquatic and Marine Research and Development (DOST-PCAMRD).

REFERENCES

Asakawa S, Himeno H, Miura K, Watanabe K. 1993. Nucleotide sequence and gene organization of the starfish *Asterina pectinifera* mitochondrial genome. Curr Genet 15(3): 193-206.

Felseinstein J., 1993. PHYLIP: Phylogeny inference package. Ver. 3.5.c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Miller M. 1991. Tools for Population Genetics Analyses (TFPGA) 1.3. A Windows program for the analysis of allozyme and molecular population genetic data. Software distributed by the author.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Ovenden JR. 1990. Mitochondrial DNA and marine stock assessment: a review. Aust J Mar Freshw Res 41: 835-853.

Richardson BJ, Baverstock PR, Adams M. 1986. Allozyme electrophoresis. A Handbook for Animal Systematics and Population Studies. Australia: Academic Press. 410 p.

Shaw CR, Prasad R. 1970. Starch gel electrophoresis of enzymes- a compilation of recipes. Biochem Genet 4: 297-320.

Steiner JJ, et al. 1995. A rapid one-tube genomic DNA extraction process for PCR and RAPD analyses. Nucl Acid Res 23: 2569-2570.

Swofford DL, Selander RB. 1981. BIOSYS-1: Fortran program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J Hered 72: 281-283.

Waples RS. 1987. A multispecies approach to the analysis of gene flow in marine shore fishes. Evolution 41: 385-400.

Ward RD, Grewe PM. 1995. Appraisal of molecular genetic techniques in fisheries. In: Carvalho JR, Pitcher TJ, editors. Molecular genetics in fisheries. London: Chapman and Hall.

Weir BS, Cockerham CC. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38: 1358-1370.

Williams ST. 1992. Methods for the analysis of genetic variation in the starfish, *Linckia laevigata*, using allozyme electrophoresis. AIMS Rep 6: 34.

Williams ST, Benzie JAH. 1993. Genetic consequences of long larval life in the starfish *Linckia laevigata* (Echinodermata: Asteroidea) on the Great Barrier Reef. Mar Biol 117: 71-77.

Williams ST, Benzie JAH. 1996. Genetic uniformity of widely separated populations of the coral reef starfish *Linckia laevigata* from the East Indian and West Pacific Oceans, revealed by allozyme electrophoresis. Mar Biol 126: 99-107.

Williams ST, Benzie JAH. 1997. Indo-West Pacific patterns of genetic differentiation in the high-dispersal starfish *Linckia laevigata*. Mol Ecol 6: 559-573.

Williams ST, Benzie JAH. 1998. Evidence of a biogeographic break between populations of a high dispersal starfish: congruent regions within the Indo-West Pacific defined by color morphs, mtDNA and allozyme data. Evolution 52: 87-99.

Yamaguchi M. 1977. Population structure, spawning and growth of the coral reef asteroid *Linckia laevigata* (Linneaus). Pac Sci 31: 13-30.