Genetic relationship of *Linckia laevigata* color morphs in the Kalayaan Islands Group, western Philippines: preliminary evidence

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ABSTRACT

Analysis of allozyme data and nucleotide sequences for a region of the mitochondrial cytochrome oxidase I (COI) gene was employed to elucidate genetic relationships among *Linckia laevigata* color morphs. Allozyme variation at 8 polymorphic loci was examined for six reef populations of *L. laevigata*, representing blue and orange morph populations collected from the Kalayaan Islands Group (KIG), South China Sea, and Tubbataha Reef, Sulu Sea. Analysis revealed grouping of populations according to color morph, with significant genetic differentiation detected between blue and orange morph populations (Fst=0.1149). In two sites where blue and orange morphs are sympatric (Panata and NE Investigator shoal), significant genetic differentiation was detected, possibly due to reproductive isolation among morphs. Notably, allele frequency shifts were observed between blue and orange morph populations at three loci, HK, PGM, and LP-1, although there was no fixation for alternative alleles. Preliminary analysis of nucleotide sequences for a limited number of *L. laevigata* collected from Panata Island reveal genetic patterns congruent with those obtained from allozyme data. Neighbor-joining analysis of sequence data reveal divergence of blue morphs from orange or mix-color morphs. Genetic differentiation of blue and orange morphs in the KIG are congruent with observed genetic patterns of *L. laevigata* color morphs across larger spatial scales, between the Indian and Pacific Ocean. In addition, fine-scale population genetic structure of *L. laevigata* in the South China Sea and Sulu Sea was revealed. Factors which may contribute to the observed fine-scale genetic patchiness are discussed.

Keywords Allozymes, mtDNA sequence, Color morphs, Kalayaan Islands Group, Biogeography, Population genetics

Introduction

The Kalayaan Islands Group (KIG) is a cluster of islets and shoal reefs situated in the South China Sea, west of Palawan, Philippines. The KIG, which is primarily a platform or patch reef formed from exposed fringing reefs, is considered to be rich fishing grounds, and is characterized by substantial diversity of marine species (Sandalo 1996). In addition, the KIG, which forms part of the Spratlys chain of islands, is believed to be a potential source of larval recruits in the area.

Linckia laevigata is a coral reef starfish found ubiquitously among shallow reef areas in the KIG. On a larger geographic scale, *L. laevigata* is widely distributed throughout the Indo-West Pacific. The species is a broadcast spawner, with larvae having a potential for long-distance planktonic dispersal, due to its relatively long larval duration (28 days, in laboratory trials) (Yamaguchi 1977). Its wide distribution and potential for extensive larval dispersal suggest that larval exchange or gene flow across large geographic distances may not be uncommon (Slatkin 1987). Scheltema and Williams (1983) report that larval duration, hence potential larval dispersal period, is inversely proportional to the level of genetic differentiation. However despite the potential of marine organisms for larval dispersal over vast geographical scales, recent studies on marine invertebrate species have shown highly significant genetic structuring among populations (Johnson and Black 1984, 1991, Watts et al. 1990, Burnett et al. 1994).

The presence of different color morphs of *L. laevigata* has been previously reported. A royal-blue morph is predominant in the Western Pacific, as well as in Western Australia. An orange/apricot morph is predominant in the Indian Ocean, although other color variants (salmon, cream, tan, yellow) have been observed (reviewed in Williams and Benzie 1998). Genetic studies of *L. laevigata* reveal genetic discontinuity between Indian and Pacific ocean populations as well as significant genetic break between the two major color morphs (Williams and Benzie 1998). Similar patterns of genetic discontinuity have been reported for other marine species (Lacson and Clark 1995, McMillan and Palumbi 1995, Lavery et al. 1995, Benzie 1999). The occurrence of concordant patterns of significant genetic discontinuity among Indian and Pacific Ocean populations of these marine species has been put forward as evidence for the existence of a biogeographic break between the two major ocean basins. The highly significant genetic divergence of *L. laevigata* color morphs demand an explanation why mechanisms of such structures occur. Even if these organisms are highly dispersed, various well known evolutionary mechanisms can operate within and between population to result in genetic divergence. These mechanims include mate preference, habitat specialization, spawning syncrony, fertilization and hybridization patterns, all of which can lead to the evolution of reproductive isolation (Palumbi 1994). Though a link between reproductive isolation and genetic divergence among populations is still unknown, recent interest has been placed in studying genetic divergence of loci that are strongly involved in reproductive isolation and species recognition.

This study focused on the color morphs of *L. laevigata* observed in the Kalayaan Islands Group. Morphs can be classified into pure blue, pure-orange or mixed-color individuals where the oral surface is predominantly blue and the aboral surface is predominantly orange. At two sites in the KIG, at Panata Island and Northeast Investigator shoal, color morphs are sympatric.

Using allozymes as initial genetic markers for defining population divergence among morphs, mtDNA sequence data was also used to further validate the allozyme patterns obtained. Thus, the objective of this paper is to determine the genetic relationship of *L. laevigata* color morphs in the KIG using allozyme markers and preliminary analyses of DNA nucleotide sequence data in the mitochondrial COI region.

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Methods

Samples

Color morphs of *L. laevigata* were collected between 1998 and 1999 from three sites in the Kalayaan Islands Group (Pagasa, Panata, and Northeast Investigator Shoal) and one site in the Sulu Sea (South Islet, Tubbataha Reef) (Fig. 1). Individuals were classified into three color morphs: pure blue, pure orange, and a combination of blue and orange (mixed-color). The South Islet population from the Tubbataha Reef was included as an outgroup population, however only blue morphs were found at this site (pure blue N=29). The pyloric caeca was dissected from *L. laevigata* samples, flash-frozen in liquid nitrogen, and stored at –70^{o} C prior to allozyme electrophoresis or DNA extraction.

Allozyme Electrophoresis

Pure blue and pure orange morphs were analyzed from the four sites sampled. These samples were designated into six groups: pure blue (Panata blue, NE Investigator blue, and S Islet blue) and pure orange (Panata orange, Pagasa orange, and NE Investigator orange). Enzymes were extracted from pyloric caeca tissues by homogenization (0.04% mercaptoethanol with bromophenol blue). Electrophoresis was carried out on horizontal starch gels following methods described in Williams (1992). Enzymes were detected with histochemical staining following procedures in Shaw and Prasad (1970). Of the 11 loci resolved, five polymorphic loci were previously employed for *L. laevigata* by Williams and Benzie (1993): glucosephosphate isomerase (GPI, 5.3.1.9), hexokinase (HK, 2.7.1.1.), superoxide dismutase (SOD, 1.15.1.1), peptidase, leu-pro substrate (LP, 3.4.11.13), peptidase, leu-tyr substrate (LT-1, 3.4.*.*). Six additional loci were identified in this study, of which three were polymorphic: isocitric dehydrogenase (IDHP, 1.1.1.42), phosphoglucomutase (PGM, 5.4.2.2), mannosphosphate isomerase (MPI, 5.3.1.8.); and three monomorphic: malate dehydrogenase (MDH, 1.1.1.37), peptidase leu-gly-gly substrate (LGG-2, 3.4.*.*), and LT-2. Isozymes coded by separate loci were numbered in order of decreasing anodal mobility. Electromorphs were equated with alleles and coded in order of decreasing anodal mobility.

DNA Extraction, PCR Amplification and Sequencing

Total DNA was extracted from *L. laevigata* pyloric caeca tissue following a rapid, one-step extraction procedure (Steiner et al. 1995). Crude DNA extracts were purified and concentrated following standard protocols (Sambrook et al. 1989). A fragment of the mitochondrial cytochrome oxidase I gene (COI) was amplified from total DNA extracts using the primers COIEF (5' ATAATGATAGGAGGRTTTGG 3') and COIER (5' GCTCGTGTRTCTACRTCCAT 3') (Arndt et al. 1996). Approximately 25-50 ng of total DNA was used as template for the amplification reactions. The PCR mixture consisted of 500 ug bovine serum albumin, 0.2 mM dNTPs, 3.0 mM MgCl2, 5 um of each primer, and 1.0 unit Taq polymerase (GIBCO-BRL) and accompanying PCR buffer, in a final volume of 10 ul. Amplification was carried out using the following thermal profile on an AirThermal Cycler (Idaho Technology): initial denaturation at 94 ^{o} C, 90 sec; 30 cycles of denaturation at 94 ^{o} C, 1 sec; annealing at 55 ^{o} C, 1 sec; and elongation at 72 ^{o} C, 30 sec, followed by a final elongation at 72 ^{o} C, 120 sec. PCR products were visualized following electrophoresis (1.0% agarose gel) and staining with ethidium bromide. PCR products from eight *L. laevigata* samples, representing the pure blue (N=2), pure orange (N=3), and mix-color or orange-blue morphs (N=3) were sequenced. PCR products were purified (Concert Purification Kit, GIBCO-BRL), and resuspended in sterile distilled water. PCR products and primers COIEF and COIER were sent to Davis Sequencing (Davis, California, USA), where automated DNA sequencing was performed using ABI PRISM® DyeDeoxy ® terminator cycle sequencing.

Statistical analysis

Allozyme data. Allele frequencies, measures of genetic variability, Nei's unbiased minimum genetic distance (Nei 1978), and pairwise comparison of genetic distance values using the UPGMA algorithm, were calculated from genotype data using the BIOSYS-1 package (Swofford and Selander 1981). Deviations of allele frequency distributions from expectations under Hardy Weinberg Equilibrium (HWE) were evaluated by a chi-square test, with pooling of rare alleles, and subsequent Bonferroni correction for multiple tests (Lessios 1992). Genetic differentiation was estimated using Wright's (1978) F-statistics, which partitions the total genetic variance into within-population (Fis) and between population (Fst) components. F-statistics were calculated using the TFPGA program (Miller 1991), where Fst and Fis were calculated for each locus and averaged across all loci using the method of Weir and Cockerham (1984), with jackknifing to obtain an unbiased estimate. F-statistics were calculated within and between populations of both blue and orange morphs. The significance of Fst values within and between blue and orange morph populations was evaluated using a contingency chi-square test according to the equations in Waples (1987). The number of effective migrants (Nem) was estimated from jackknifed Fst values using an island model of population structure, where genetic divergence is related to gene flow by the formula Nem=[(1/ Fst)-1]/4.

DNA sequence data. All sequence data were obtained by double-stranded sequencing. Alignment of nucleotide sequences was performed using ClustalW (Thompson et al. 1994), and. a 587-base region of the COI gene from 8 *L. laevigata* collected from Panata Island were unambiguously aligned. Nucleotide diversity and genetic variance among the sequences were calculated using the program DNASp (Rozas and Rozas 1999). Genetic distance data was calculated employing the Kimura-2-parameter model with genetic affinities inferred from the resulting distance matrix by neighbor-joining analysis, as implemented in the PHYLIP software package ver 3.5 (Felsenstein 1993). Panata Island samples were then compared with *L. laevigata* color morphs collected from other sites in the Indo-Pacific (Williams 2000) to be able to compare sequences with previously existing DNA data: sequences samples collected from Bolinao, Philippines (Phil2-6; accession nos. AF18798, AF187900-03), Thailand (Thai1, AF187913; Thai4, AF187915; Thai5, AF187916), South Africa (SA2, AF187919; SA3, AF187920), and Western Australia (WA3, AF187906). Sequences from another Linckia species, *L. guildingii* (LgCV2, AF187945; LgCV3, AF187946) were used as outgroups.

Results

Allozyme

L. laevigata morphs collected from 4 sites in the South China Sea were studied, and designated into 6 groups representing pure blue and pure orange individuals. Allele frequencies and genetic variability estimates at 8 polymorphic loci are presented (Table 1) (data for three monomorphic loci not included in the table). Average heterozygosity values (direct-count estimates) ranged from 0.1680 (Panata blue) to 0.3072 (South Islet blue). Average heterozygosity over all loci was slightly higher for the orange morph groups (mean Ho=0.2454) than the blue morph groups (mean Ho=0.2281). Nei's unbiased minimum genetic distance (D), ranged from 0.002 to 0.0874 (Table 2). Pairwise comparison of D values reveal that genetic distance among blue populations (mean D=0.007) and among orange populations (mean D=0.0226), are both smaller relative to the mean D between blue and orange populations (mean D=0.0513). The genetic relationship among the six groups is illustrated in a dendrogram generated using the UPGMA algorithm. Blue morph and orange morph populations form two separate groups (Fig. 2).

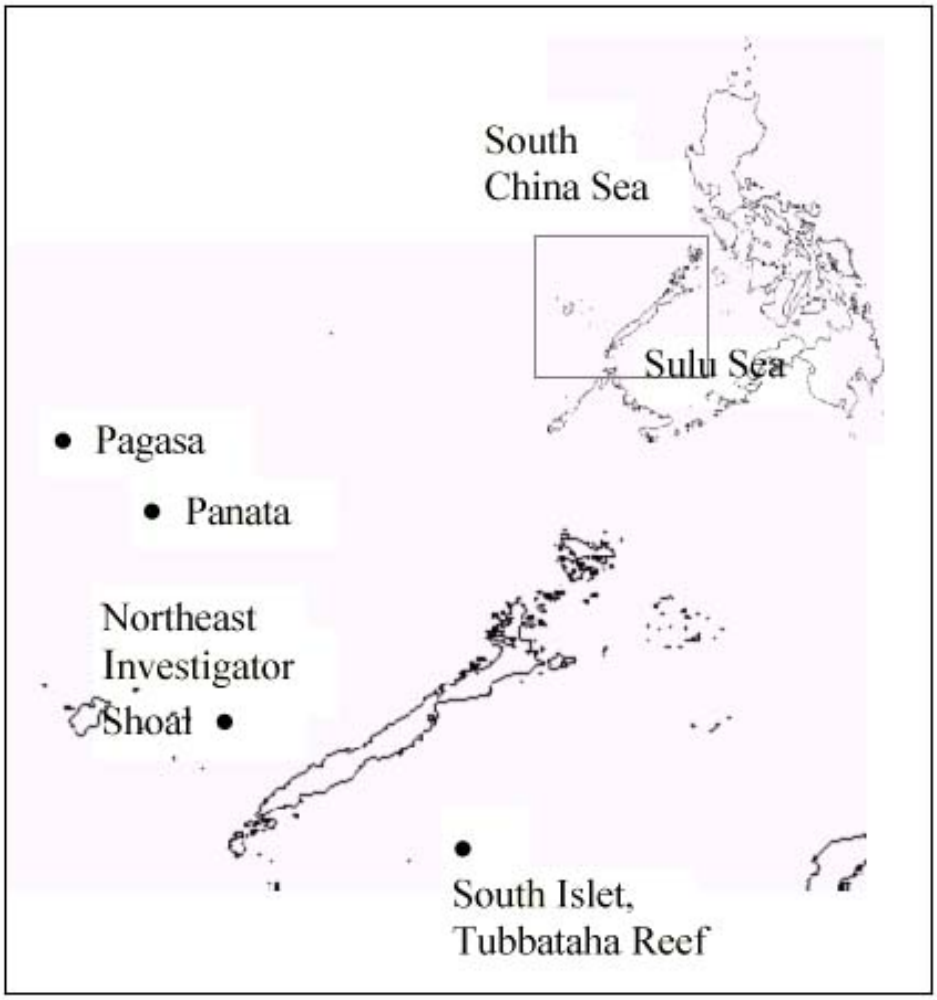

Fig. 1. Map showing collection sites for *L. laevigata* in the Kalayaan Islands Group, South China Sea, and Sulu Sea (inset, enlarged).

Table 1. Allele frequencies at 8 polymorphic loci for 6 groups of *Linckia laevigata* representing blue and orange morphs in the Kalayaan Islands Group, western Philippines. Sample sizes are in parentheses. Monomorphic loci not included.

Locus/Allele	Panata Blue (26)	NE Inves Blue (30)	S. Islet Blue (29)	Pag-asa Orange (36)	Panata Orange (29)	NE Inves Orange (13)
HK-1						
117	0.3462	0.1833	0.2931	-	0.0345	-
111	0.4615	0.5500	0.5862	0.0139	0.1034	0.0385
100	0.1923	0.2667	0.1207	0.2500	0.5690	0.2692
91	-	-	-	0.7083	0.1379	0.5000
87	-	-	-	0.0278	0.1552	0.1923
SOD-1						
150	0.0192	0.0500	0.0517	0.0833	0.0862	-
100	0.9615	0.9167	0.8793	0.8472	0.9138	0.9231
80	0.0192	0.0333	0.0690	0.0694	0.0000	0.0769
IDHP-1						
110	0.0192	0.0333	0.0862	0.0417	0.0517	0.0385
100	0.9231	0.8833	0.7241	0.7917	0.8103	0.9615
95	0.0000	0.0167	0.1897	0.0833	0.0172	-
89	0.0577	0.0667	0.0000	0.0833	0.1034	-
85	-	-	-	-	0.0172	-
PGM-1						
110	0.3077	0.2167	0.3276	0.0139	0.0690	0.0385
100	0.5000	0.5167	0.5517	0.2639	0.5345	0.2692
95	0.1923	0.2667	0.1207	0.7222	0.1724	0.5385
90	-	-	-	-	0.2241	0.1538
MPI-1						
130	0.0192	0.0333	0.0862	0.0417	0.0517	0.0385
100	0.9231	0.8667	0.7414	0.8056	0.7586	0.9231
92	-	0.0167	0.1724	0.1111	0.0690	0.0385
87	0.0577	0.0833	-	0.0417	0.1034	-
85	-	-	-	-	0.0172	-
LT-1						
125	0.2500	0.1833	0.2931	0.3333	0.2414	0.3077
110	-	0.0667	-	0.0278	-	-
100	0.7500	0.7333	0.7069	0.6389	0.7586	0.6154
89	-	0.0167	-	-	-	0.0769
LP-1						
120	0.0769	0.0167	0.0517	0.1111	0.0862	0.2692
117	0.2308	0.0333	0.0172	0.4306	0.4483	0.2692
100	0.5192	0.6833	0.7414	0.2361	0.3448	0.3846
92	0.1731	0.2000	0.0690	0.2222	0.1207	0.0769
87	-	0.0667	0.1207	-	-	-
GPI-1						
120	-	-	-	0.0417	0.0517	0.0385
110	0.0385	0.0333	0.0862	-	-	-
100	0.9038	0.8833	0.7414	0.8611	0.8103	0.9615
95	-	-	0.1742	0.0556	0.0172	-
92	0.0577	0.0833	-	0.0417	0.1034	-
87	-	-	-	-	0.0172	-
Mean heterozygosity (direct-count)	0.1680	0.2091	0.3072	0.2273	0.2853	0.2238
Mean heterozygosity (Nei's unbiased estimate)	0.2551	0.2674	0.3136	0.2942	0.3180	0.2644
% polymorphic loci	63.63	72.72	72.72	72.72	72.72	54.54

Table 2. Matrix of Nei's (1978) unbiased minimum distance values for 6 groups representing blue and orange morphs of *Linckia laevigata* in the Kalayaan Islands Group, western Philippines. Comparisons between blue morph populations and orange morph populations are in boldface.

Population	1	2	3	4	5	6
1. Panata blue	-					
2. NEInv blue	0.0020	-				
3. Sislet blue	0.0121	0.0080	-			
4. Pagasa orange	**0.0621**	**0.0675**	**0.0875**	-		
5. Panata orange	**0.0254**	**0.0306**	**0.0472**	0.0388	-	
6. NEInv orange	**0.0367**	**0.0415**	**0.0630**	0.0071	0.0219	-

No significant within-population (Fis) differentiation was detected. Genetic variance between the 6 groups (Fst), averaged over all loci (Table 3), indicate significant genetic differentiation (Fst = 0.1149, $p<0.001$). Per locus Fst values are significant for 5 out of 8 loci: IDHP and GPI-1 ($p<0.005$), HK, PGM, and LP-1 ($p<0.001$). Moreover, Fst values of blue and orange groups from Panata and NEInvestigator were significant (Table 3). Three loci contribute to this result: HK, PGM, and LP-1, which exhibited shifts in the frequency of the most common allele between blue and orange morphs, although no fixation for alternative alleles was observed.

Taken together, these results suggest genetic differentiation and possible reproductive isolation among blue and orange morphs. Indication of fine-scale substructure was investigated between populations of the same color morph (Table 3). Pairwise analysis of genetic variability among blue morph populations indicate significant genetic differentiation between KIG (Panata and NEInvestigator) and South Islet, Tubbataha Reef (overall Fst = 0.0208, $p<0.001$), with 4 loci contributing to this observed differentiation (IDHP, HK, PGM, and GPI). Likewise, significant genetic structuring was observed among orange morph populations in the KIG,with the three populations (Pagasa, NEInvestigator, and Panata) genetically distinct from each other (overall Fst = 0.0828, $p<0.001$), with 4 loci contributing to differentiation (IDHP, HK, PGM, and GPI).

Table 3. F-statistics of blue morph and orange morph groups of *Linckia laevigata*. Overall FST and FIS values presented are jackknifed estimates over all loci. The number of effective migrants (Nem) was calculated using the island model.

	No. of groups	F_{IS}	F_{ST}	Nem
Between color morph groups				
Blue vs orange groups	6	0.1902	0.1149***	1.9
Panata blue vs orange	2	0.2206	0.0811***	2.8
NEInvestigator blue vs orange	2	0.2214	0.1370***	1.6
Within blue morph group				
KIG sites	2	0.3049	0.0027	92.3
KIG + South Islet	3	0.2053	0.0208**	11.8
Within orange morph group				
KIG sites	3	0.1861	0.0828***	2.8
Pag-asa + NEInvestigator	2	0.2375	0.0178***	13.8
Pag-asa + Panata	2	0.1957	0.1149***	1.9
NEInvestigator + Panata	2	0.1490	0.0631***	3.7

mtDNA sequence

Preliminary analysis of 587 bases of the mitochondrial cytochrome oxidase I gene amplified from a limited number of *L. laevigata* color morphs reveal broadly consistent genetic patterns with those obtained from allozyme markers. Firstly, greater nucleotide diversity was noted for orange and mix-color sequences (π= 1.4% and 0.3% respectively) relative to blue morph sequences. Nucleotide sequence of the pure blue morphs (N=2) (PB3 and PB12) were identical, while sequences of each of the orange morphs (N=3) (PO29, PO30, PO31) and mix-color morphs (N=3) (OB23, OB24, OB25) were unique. Secondly, pairwise comparison of sequence diversity among Panata Island samples (data not shown) reveal that blue and orange morph comparisons have greater nucleotide sequence divergence (dxy = 0.01772) relative to mixed color and orange morph comparisons (dxy = 0.00886). No fixed nucleotide differences/substitutions were observed between mixed-color and orange morphs, while a total of 8 fixed differences were

observed between blue and mix-color/orange morphs. Of the 8 variable sites, 7 substitutions are transitions and the remaining substitution is a transversion. Neighbor-joining analysis was conducted on an expanded data set, with additional nucleotide *L. laevigata* sequences from Southwestern Pacific and Indian Ocean samples (Willliams 2000).

This was performed to compare Panata sequences to DNA sequence data reported previously, thus determine genetic affinities of Panata Island samples with samples previously identified as belonging to two separate clades: (1) a Southwestern Pacific clade (Phil2, Phil5, GBR2, Guam5) and (2) an Indian Ocean clade (SA2, SA3, Thai1, Thai4, Thai5, Phil3, Phil4, Phil6, WA3). *L. laevigata* from Panata island occurred in two separate clades (Figure 3). Preliminary analysis of genetic relationships among these DNA sequence samples reveal clustering of individuals according to color. The pure blue Panata Island morphs clustered with Southwestern Pacific clade samples, while pure orange and mix-color Panata Island morphs clustered with the Indian Ocean clade samples.

Discussion

Population subdivision

Previous studies on *L. laevigata* (Williams and Benzie 1998) and *A. planci* have demonstrated patterns of genetic differentiation congruent with color morph distribution. However, color morph populations in these studies were separated by large geographic distances, between Indian and Pacific Ocean populations. Genetic separation has been suggested as resulting from climatological and/or geological processes that may have restricted dispersal of marine species between the Indian and Pacific Oceans (Williams and Benzie 1998). Dispersal across this historical barrier, i.e. during periods of high sea-level, would account for the occurrence of orange morphs in the South China Sea and isolated sites in the Western Pacific, creating a region of overlap of the color morphs in the Indo-Malay region and Northwest Pacific (Willliams 2000). In this study, allozyme analysis of *L. laevigata* color morphs in the KIG has demonstrated significant genetic differentiation between blue and orange morph populations, indicating that genetic distinction of color morphs is maintained even in sympatry. At two sites in the KIG where blue and orange morphs are sympatric (Panata island and NEInvestigator shoal), genetic distinction based from allozyme markers is evidenced by the highly significant Fst values between blue and orange populations (Panata: Fst =0.0811; NEInvestigator Fst =0.1370). These Fst values between sympatric populations in the KIG are comparable to Fst values reported by Williams and Benzie (1998) between blue and orange morphs across the Indo-Pacific. Fst values for Indo-Pacific populations range from Fst=0.083 (all orange morph vs. all blue morph) to Fst=0.106 (South Africa orange morph vs. blue morphs from 8 sites in the Western Pacific and Western Australia). Nei's unbiased minimum genetic distance (D) between sympatric color morphs in the KIG was also high (Panata D=0.0254; NEInvestigator D=0.0415). Mean Nei's D between blue and orange morphs across the Indo-Pacific is 0.1178 (Williams and Benzie 1998). Notably, preliminary genetic patterns inferred from mitochondrial DNA sequence data, although from a limited number of individuals (N=8), is consistent with allozyme analyses in the observed genetic discontinuity between color morphs. Additionally, separation of Panata island samples into two groups was evident,where blue individuals clustered with a southwestern Pacific clade and orange and mix-color individuals clustered with an Indian Ocean clade. Patterns of genetic structuring of color morphs of *L. laevigata* can thus be delineated using two molecular markers: allozymes and mtDNA. Many studies reported in the literature substantially give importance to genetic studies of marine organisms using multiple markers.

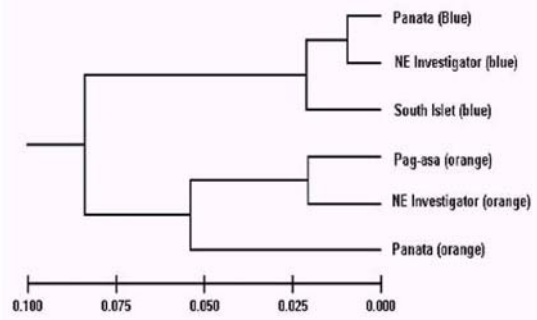

Fig. 2. Dendrogram illustrating genetic relationships among 6 populations of *Linckia laevigata*, representing blue and orange color morphs, from the KIG, South China Sea and Sulu Sea, based on allozyme data. Nei's unbiased genetic distances (Nei 1978) were clustered using the UPGMA method.

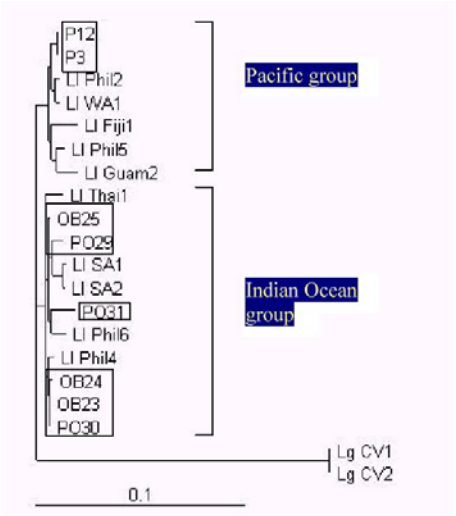

Fig. 3. Neighbor-joining tree based on Kimura-2- parameter estimates of nucleotide sequence divergence among *L. laevigata* color morphs from the Kalayaan Islands Group and various locations in the Indo-Pacific. Color morphs from Panata island are boxed, sequence labels are as in the text. Color morphs from other Indo-Pacific sites (Williams 2000) are identified: (1) orange morphs from South Africa (SA), Thailand (Thai) and Philippines (Phil3); (2) blue morphs from the Philippines (Phil2), Western Australia (WA3), Guam (Guam5), and the Great Barrier Reef (GBR2); and (3) mix color individuals from Philippines (Phil3-Phil6). *Linckia guildingi* (LgCV) was used as an outgroup.

The relatively long larval duration of *L. laevigata* and its widespread distribution indicates potential for extensive larval dispersal, such that gene flow over long distances may be common (Slatkin 1987). However, the potential for extensive gene flow may not always be realized, as demonstrated by observed fine-scale population genetic structure of *L. laevigata* in the KIG. Similarly, fine-scale genetic structuring was observed for *L. laevigata* populations from various Palawan shelf and shoal reefs (Meñez et al., unpublished data). This was also true for populations of the giant clam, *Tridacna crocea* (Meñez et al., unpublished data) and a reef fish, *Chromis margaritifer* (Endriga et al. unpublished data). Several factors may contribute to the observed genetic patchiness in *L. laevigata* populations in the KIG. Physical barriers to larval dispersal or conditions affecting larval recruitment success (i.e. variable and complex hydrographic regimes) play a role in the genetic structuring of reef communities. In addition, other parameters, i.e. diverse reef morphologies may provide variable habitats (microhabitats), contributing to 'patchy' recruitment of reef species. Compared with the Great Barrier Reef (GBR) which is a highly connected reef network where suitable habitats are more or less continuously available, the reef systems in the KIG are relatively isolated, reducing effective larval dispersal and gene mixing over wide geographic scales. Alternatively, the observed genetic patchiness may be reflective of the stochastic nature of larval dispersal and recruitment among populations (Watts et al. 1990, Palumbi 1997).

A mechanism for reproductive isolation?

The genetic discontinuity of sympatric populations of *L. laevigata* from Panata and NEinvestigator in the KIG based from allozyme markers is of particular interest. Genetic differentiation of sympatric populations is usually considered as strong evidence to determine the existence of species-level morphological variation (Palumbi 1997). Reduced gene flow between morphs, may indicate the possibility that blue and orange morphs *of L. laevigata* may represent separate species or subspecies. However, available genetic data are limited, and allozyme genetic information do not provide sufficient evidence of reproductive isolation (i.e. presence of fixed allele frequency differences) between color morphs. Additional evidence, such as reproductive compatibility between morphs and genetic data from mixed-color morphs, would be valuable in delineating whether the observed genetic divergence between color morphs is indicative of population- or species-level morphological variation. The question of species- or population-level variation nothwithstanding, restricted gene flow between sympatric populations of *L. laevigata* color morphs is evident. Cursory observations during field collections indicate that blue morphs are more abundant in shallow water (0-5 m), while orange morphs are usually found at greater depths (5-20 m). Experimental data for a seastar, *Coscinasterias muricata*, suggests water depth as a factor affecting fertilization success, with fertilization rates decreasing exponentially with distance from gamete sources (Babcock et al. 2000). A case of habitat specializaation may be restricting gene flow between orange and blue morphs of *L. laevigata*. In addition, other intrinsic factors may be involved in restricting gene flow, such as non-synchronous spawning periods, environmental tolerance and gamete incompatibility, information for which are not completely available to date for *L. laevigata* color morphs.

Conclusion

Allozyme data and preliminary evidences based from mtDNA sequence analysis point to significant genetic structuring between blue and orange morph populations of *L. laevigata*. Using two genetic markers, data showed interesting patterns of fine-scale genetic substructuring of color morph populations based from allozymes and high mtDNA COI sequence divergence between color morphs sampled from sites in the KIG and Sulu Sea. This study is an initial effort to identify patterns of genetic change, therefore additional allozyme analysis of mixed-color morphs and nucleotide sequence data are necessary to further elucidate the genetic relationships and possible reproductive isolation between blue and orange morphs in the South China Sea. These will provide data on the estimation of species divergence times and calibration of molecular clock between color morphs. Such information will be valuable in providing additional insights to the biogeographic history of the species in the region, and may provide inferences on mechanisms of larval dispersal, reproductive isolation and recruitment of morph populations, and their role in shaping the genetic structure and speciation of *L. laevigata* in the region.

Acknowledgements The authors thank SCS and KIG Project cruise participants for sample collection, PM Aliño and JAH Benzie for comments and discussions. Financial support was provided by the Department of Science and Technology (Philippines, South China Sea Project). Funding support for travel, from the Commission on Higher Education (R. Magsino and R. Ravago) and the Lucille-Packard Foundation through ISRS (R. Magsino) is also gratefully acknowledged. This is Marine Science Institute Contribution No. 315.

References

Arndt A, Marquez C, Lambert P, Smith MJ (1996) Molecular phylogeny of eastern Pacific sea cucumbers (Echinodermata:Holothuroidea) based on mitochondrial DNA sequence. Molec Phylog Evolut 6:425-437.

Babcock RE, Franke JS, Barr N (2000) Does spawning depth affect fertilization rates? Experimental data from the sea star *Coscinasterias muricata*. Mar Freshwat Res 1:55-61

Benzie JAH (1999) Major genetic differences between crown-of-thorns starfish (*Acanthaster planci*) populations in the Indian and Pacific Oceans. Evolution 53(6):1782-1795

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.

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Johnson MS, Black R (1984) Pattern beneath the chaos: the effect of recruitment on genetic patchiness in an intertidal limpet. Evolution 38(6):1371-1383

Johnson MS, Black R (1991) Genetic subdivision of the intertidal snail Bembicium vittatum varies with habitat in the Houtman Abrolhos Islands, Western Australia. Heredity 67:205-213

Lacson JM, Clark S (1995) Genetic divergence of Maldivian and Micronesian demes of the damselfishes *Stegastes nigricans*, *Chrysiptera biocellata*, *C. glauca and C. leucopoma* (Pomacentridae). Mar Biol 121:585-590

Lavery S, Moritz C, Fielder DR (1995) Changing patterns of population structure and gene flow at different spatial scales in *Birgus latro* (the coconut crab). Heredity 74:531-541

Lessios HA (1992) Testing electrophoretic data for agreement with Hardy-Weinberg expectations. Mar Biol 112:517-523

McMillan WO, Palumbi SR (1995) Concordant evolutionary patterns among Indo-West Pacific butterfly fishes. Proc R Soc Lond 260:229-236

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

Moberg PE and Burton RS (2000) Genetic heterogeneity among adult and recruit red sea urchins, *Strongylocentrotus franciscanus*. Mar Biol 136:773-784

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

Palumbi SR (1994) Genetic divergence, reproductive isolation, and marine speciation. Annu Rev Ecol Syst 25:547-572

Palumbi SR (1997) Molecular biogeography of the Pacific. Coral Reefs 16, Suppl: S47-S52.

Rozas J, Rozas R (1999) DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15: 174-175

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2 nd ed.). Cold Spring Harbor Press, New York: 1720 pp

Sandalo AC (1996) Overview and status of fisheries and coastal resources management in Palawan, Philippines. In: Pido M.D et al. (eds.) The management systems of marine fisheries and other coastal resources in Palawan, Philippines: Concepts, experiences and lessons. ICLARM and PCSD : 2-9

Scheltema RS, Williams IP (1983) Long distance dispersal of planktonic larvae and the biogeography and evolution of some Polynesian and Western Pacific mollusks. Bull Mar Sci 33(3):545-565

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

Slatkin M (1987) Gene flow and the geographic structure of natural populations. Science 236:787-792

Steiner JJ, Poklemba CJ, Fjellstrom RG, Elliot LF (1995) A rapid one-tube genomic DNA extraction process for PCR and RAPD analyses. Nucl Acids Res 23:2569-2570

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

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 22: 4673-4680

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

Watts RJ, Johnson MS, Black R (1990) Effects of recruitment on genetic patchiness in the urchin Echinometra mathaei in Western Australia. Mar Biol 105:145-151

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

Williams ST (1992) Methods for analysis of genetic variation in the blue starfish, *Linckia laevigata*, using allozyme electrophoresis. AIMS, report no. 6. Townsville, Australia

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 (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(1):87-99

Williams ST (2000) Species boundaries in the starfish genus *Linckia*. Marine Biology 136:137-148

Wright S (1978) Evolution and the genetics of populations. Vol. 4. Variability in and among natural populations. Univ. of Chicago Press, Chicago

Yamaguchi M (1977) Population structure, spawning, and growth of the coral reef asteroid *Linckia laevigata* (Linnaeus).Pac Sci 31:13-21.