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Temporal genetic variation in populations of the limpet *Cellana grata* from Hong Kong shores

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Abstract Variations in the relative contributions of gene flow and spatial and temporal variation in recruitment are considered the major determinants of population genetic structure in marine organisms. Such variation can be assessed through repeated measures of the genetic structure of a species over time. To test the relative importance of these two phenomena, temporal variation in genetic composition was measured in the limpet *Cellana grata*, among four annual cohorts over 10 years at four rocky shores in Hong Kong. A total of 408 limpets, comprising individuals from 1998, 1999, 2006 and 2007 cohorts were screened for genetic variation using five microsatellite loci. Minor but significant genetic differentiation was detected among samples from the 1998/1999 collection ($F_{ST} = 0.0023$), but there was no significant differentiation among the 2006/2007 collection ($F_{ST} = 0.0008$). Partitioning of genetic variation among shores was also significant in 1998/1999 but not in the 2006/2007 collection, although there was no correlation between genetic and geographic distances. There was no significant difference between collections made in 1998/1999 and 2006/2007. This lack of clear structure implies a high level of gene flow, but differentiation with time may be the result of stochastic recruitment variation among shores. Estimates of effective population size were not high (599, 95% C.L. 352–11397), suggesting the potential susceptibility of the populations to genetic drift, although a significant bottleneck effect was not detected. These findings indicate that genetic structuring between populations of *C. grata* in space and time may result from spatio-temporal variation in recruitment, but the potential development of biologically significant differentiation is suppressed by a lack of consistency in recruitment variability and high connectivity among shores.

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Introduction

The transition from the pelagic larval to benthic life stage in many marine invertebrates is often the most vulnerable stage within the life cycle of a species and has been the focus of "supply-side ecology" (Underwood and Fairweather 1989). The unpredictable and often stochastic chances of survival often result in highly fluctuating numbers of successful progenies that survive to the settlement and finally recruitment stage (Hedgecock 1994). This variability is one of the crucial factors governing the on-shore population structure of many species of marine invertebrates (Morgan 2001). To complicate this picture, environmental variables also change from time to time at different spatial and temporal scales, which may further exacerbate stochastic patterns in larval supply and subsequent recruitment (Hawkins and Hartnoll 1982; Jenkins et al. 1997; McIlwain 2003; Svensson et al. 2004).

The inherent variation in larval supply is also believed to affect levels of genetic variability, which has been recorded in a variety of taxa. The term "chaotic genetic patchiness" has been used to describe genetic heterogeneity among populations over small spatial and temporal scales (Johnson and Black 1982). Such a pattern is not, however, uncommon, even in species with planktonic larvae and high dispersal ability (Johnson and Black 1984a; Pujolar et al. 2006). Different hypotheses have been proposed to explain such apparently unpatterned genetic structure. Firstly, it has been suggested that genetic heterogeneity on small spatial/temporal scales is a consequence of extremely large variance in individual reproductive success, which is a combined effect of high fecundity and sweepstakes-chance matching of reproductive activity with oceanographic conditions (Li and Hedgecock 1998; Lundy et al. 2000). In this case, recruits from each spawning event may inherit different proportions of the potential adult gene pool, leading to temporal genetic variance (Lenfant and Planes 2002; Bernal-Ramírez et al. 2003). Secondly, temporal variation in genetic composition may be a result of larvae being contributed from different source populations. Changes in environmental variables, such as the direction of current flow, may alter the source larval populations and hence cause genetic differentiation among recruits (Kordos and Burton 1993). Finally, selective mortality at pre- or post-settlement stages may shape on-shore population structure in a site or time specific manner, dependant on the extent or consistency of the selection force (Johnson and Black 1984a).

The likelihood for genetic patchiness to develop to biologically significant differentiation depends largely on the independence of the potential sub-populations, which is inversely related to their degree of connectivity. The impact on the overall population genetic structure could be short term, when the unmixed portion of the population is not robust enough to sustain a lasting genetic structure (Selkoe et al. 2006), or long term if genetic differentiation between cohorts accumulates over time (Maes et al. 2006).

Located on the South China coast, intertidal community structure in Hong Kong is highly dynamic, as reflected by spatial and temporal variation in settlement and recruitment (Huang 2001; Chan and Williams 2004; Yan et al. 2006). Such variation is likely to be linked with on-shore genetic structure (Li and Hedgecock 1998). The present study uses the nacellid limpet *Cellana grata* (Gould 1859) as a model organism to evaluate potential temporal variation in genetic structure on Hong Kong shores. *C. grata* is found in the mid-high shore on moderately exposed to exposed rocky shores and is a broadcast spawner (Williams and Morritt 1995). The length of the pelagic larval stage is unknown for this species but varies between 4 and 18 days in congeneric species (Bird et al. 2007). The recruitment period of *C. grata* is from October to July, with its peak intensity during March (Liu 1994a). In Hong Kong, *C. grata* lives for 3+ years and exhibits a predictable, annual breeding cycle (Liu 1994a; Huang 2001). Most shores support populations that composed of two to three clearly identifiable cohorts, the newly settled cohort, a 1+ year cohort that is usually the dominant cohort, and remnants of 2+ year and very occasionally 3+ year cohort (see Williams and Morritt 1995). These characteristics make it possible to collect multiple cohorts for temporal analysis of genetic structure in this species from different sites. As such, this makes *C. grata* an ideal animal for the present study as the evaluation of temporal genetic patterns requires repeated sampling from the same populations (Waples 1998). Most recent studies have, however, been confined to sampling only 2 or 3 years/spawning events (e.g. Virgilio and Abbiati 2006; Hepburn et al. 2009), which may limit the power to identify the presence of accumulated variation over years. The present study collected two groups of two successive yearly cohorts of *C. grata*, separated by 10 years from 1998 to 2007, from four shores in Hong Kong to investigate the temporal genetic structure of this limpet and to interpret possible causes of the observed patterns.

Materials and methods

Specimen collection and demographic analysis

To investigate temporal genetic structure of *Cellana grata*, samples were collected in 1999 and 2007 from four rocky shores in Hong Kong SAR, China. The shores represented the common settlement locations of the animal from the east to west of Hong Kong, namely, Tiu Chung Chau (T), Big Wave Bay (B), Beaufort Island (L) and Discovery Bay (D, Fig. 1). Over 100 limpets were randomly collected between 1.25 and 2.00 m above Chart Datum at each shore in each year. All specimens were fixed in 95% ethanol and stored at −20°C before laboratory analysis. In the laboratory, maximum shell length (±0.1 mm) was measured, and population histograms (1 mm size interval) generated for each shore. Individual cohorts were separated using Bhattacharya's method (Bhattacharya 1967) using FiSAT (FAO-ICLARM Fish Stock Assessment Tool, Gayanilo and Pauly 1997) for each shore in each year. Two of the cohorts, namely, the new settlers of the year and the settlers from the previous year, were used for subsequent analysis, resulting in four identifiable cohorts (1998, 1999, 2006 and 2007) from each shore ($\Sigma_{\text{sample}} = 4$ cohorts $\times$ 4 shores $= 16$ samples). The total number of specimens used for the genetic study was 408 (Table 1).

DNA extraction and microsatellite amplification

Genomic DNA was extracted using the Qiagen QIAamp® DNA Mini Kit (Catalogue no. 51306) according to the

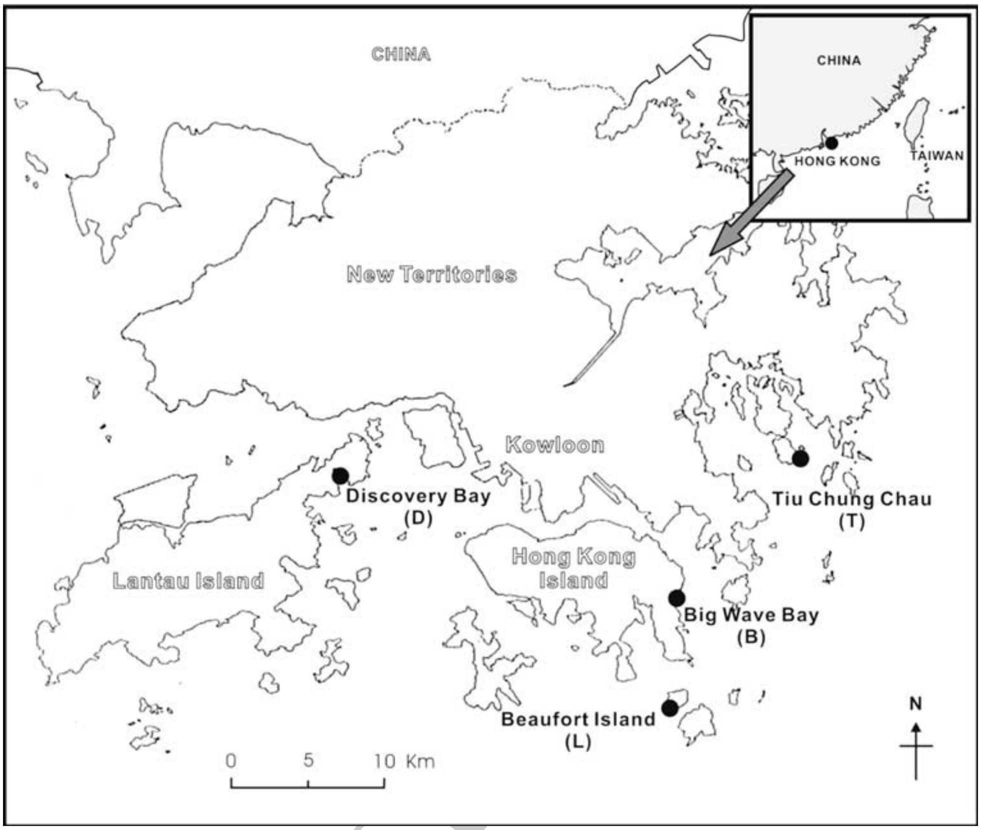

Fig. 1 *Cellana grata*. Sampling locations distributed across Hong Kong

manufacturer's protocol. DNA was eluted with 60 µl of Buffer TE and was kept at −20°C. Individuals were genotyped at five novel microsatellite loci developed (primer sequences, annealing temperatures and characters of the loci are detailed in Ng et al. 2009). Forward primers were fluorescently labeled. Polymerase chain reactions (PCR) were set up with 20 ng of genomic DNA, 0.8 unit of Taq Polymerase (Qiagen), 1.25 mM $MgCl_2$, 0.2 µM of forward and reverse primers, and 0.2 µM of each dNTPs in 20 µl reaction mixes. Reaction profiles were set at for 15 min at 95°C, 39 cycles of 1 min at 94°C, 50 s at appropriate annealing temperature, and 90 s at 72°C, and a final extension for 5 min at 72°C. Amplified products were run in a sequencing gel using an ABI 3100 automated sequencer with 400HD ROX^{TM} (Applied Biosystems) as an internal size standard. Allele sizes were scored using GeneMarker (Softgenetics).

Population genetic analysis

For each sample, allelic richness was computed for each locus using FSTAT v.2.9.3.2 (Goudet 1995). Observed (H_O) and expected (H_E) heterozygosities were calculated using GENETIX v.4.05 (Belkhir et al. 2005). F_{IS} were calculated according to Weir and Cockerham (1984). Deviation from the Hardy–Weinberg equilibrium was tested using GENEPOP v.3.4 (Raymond and Rousset 1995) using the Markov chain method (Guo and Thompson 1992). Genotypic linkage disequilibria between any two loci were tested using Markov chain and Fisher exact tests (10,000 dememorization, 1,000 batches, 5,000 iterations). The potential deviations from Hardy–Weinberg equilibrium due to the presence of null alleles were estimated using MICRO-CHECKER version 2.2.3 (van Oosterhaut et al. 2004).

As differences in genetic diversity among the samples were noted (see "Results"), evidence for recent changes in effective population size was tested. Populations that have experienced a recent reduction in their effective population size exhibit a correlative reduction in allele numbers and gene diversity (Hardy–Weinberg heterozygosity) at polymorphic loci. Allele numbers are, however, reduced faster then gene diversity, and hence observed gene diversity is higher than the expected equilibrium gene diversity in a recently bottlenecked population (Cornuet and Luikart 1996). To estimate this, heterozygosity values (H_e) for each sample were tested to investigate whether they exceeded those expected at mutation–drift equilibrium (H_{eq}) under the infinite alleles modes (IAM), strict stepwise mutation model (SMM) and 2-phase model (TPM). Computation was performed using BOTTLENECK (Cornuet and Luikart 1996), with the proportion of single-step mutations set to 70% for TPM. The null hypothesis of no difference between H_e and H_{eq} was determined using the Wilcoxon sign-rank test that provides higher power in the case of few microsatellite loci (Luikart and Cornuet 1998). The four shores for each cohort were also combined for overall estimates of potential bottlenecks in each cohort.

Genetic differentiation among samples was evaluated for the two temporal collections, i.e. 1998/1999 and 2007/2007, using F-statistics as implemented in GENETIX v.4.05. Global and pairwise F_{ST} were calculated across all the samples following Weir and Cockerham's θ (1984) and the 95% confidence intervals (C.I.) generated by 1,000 bootstrap iterations. Population differentiation was also tested by Fisher's exact tests using the Markov chain method (10,000 dememorization, 1,000 batches, 5,000 iterations) that takes into account low-frequency alleles and provides unbiased estimates of exact P values. The test was conducted both globally and between sample pairs using GENEPOP v.3.4 with the null hypothesis that "allelic distribution is identical across populations". In all tests, significance levels were corrected for multiple comparisons using a sequential Bonferroni technique with overall $\alpha = 0.05$ (Rice 1989).

Pairwise sample by sample genetic distances were calculated following Cavalli-Sforza and Edwards (1967) to generate chord distance (D_{CE}) using GENETIX v.4.05. This method makes no assumption of mutation, and distances are standardized with respect to random drift. Compared with other frequency-based genetic distances, D_{CE} offers lower sampling error and provides a more efficient mechanism to generate correct tree topologies under different demographic conditions (Takezaki and Nei 1996). The D_{CE} matrix was visualized using multidimensional scaling as implemented in PRIMER 6 (Clarke and Gorley 2006).

Partitioning of genetic variability into spatial and temporal components was performed using an analysis of molecular variance (AMOVA) in ARLEQUIN v.3.1 (Excoffier et al. 2005). For spatial analysis, genetic variability was partitioned into within shore (Φ_{SC}) and between shores (Φ_{CT}) variation. The variability components were evaluated separately for 1998/1999 and 2006/2007 collections. For temporal analysis, an AMOVA was carried out by grouping the samples by sampling time (i.e. 1998/1999 versus 2006/2007 collections) to examine whether there was significant variation in population structure over 10 years. Isolation by distance (IBD) between samples was evaluated for 1998/1999 and 2006/2007 collections using the Mantel test as implemented in GENETIX v.4.05 (10,000 permutations) by correlating linearized F_{ST} (i.e. $F_{ST}/[1-F_{ST}]$) to the hydrographical distances, which were measured as the minimal along-shore distance between shores. To evaluate the effective population size, the moments-based method of Waples (1989) was employed, which estimates N_e from the standardized variance in allele frequency between temporal samples separated by a known number of generations. The generation time was set at 4 years based on population dynamics observation that revealed the general existence of cohorts from 0+ to 3+ years for the species (Huang 2001). Samples from 1998/1999 collection were combined into one generation, and the same was applied for the 2006/2007 collection. Calculation was performed using NEESTIMATOR version 1.3 (Peel et al. 2004).

Assignment tests were conducted to estimate the probability of individuals that would be assigned to the stock origin based on genotypic data using the algorithm suggested by Cornuet et al. (1999). The probability of belonging to a group was computed for each individual, and individuals were assigned to one of the sixteen samples with the highest score using GENECLASS2 (Piry et al. 2004) under the Bayesian criteria (Rannala and Mountain 1997). A 10,000 test simulation was performed with resampling by the "leave-one-out" option that corrects for the bias of over rejection of resident individuals (Paetkau et al. 2004). The assignment rates among samples/collections were tested using ANOVA with arcsine square-root transformation.

Results

Demographic structure

Two to three distinct cohorts could be identified in all the 8 collections (4 shores × 2 collection years), with high (>2) separation indices. The mean total length of the newly settled cohort ranged from 8.7 to 12.7 mm and 18.1 to 26.0 mm for the second year cohort. A third cohort could be identified in Tiu Chung Chau (both in 1999 and 2007), Beaufort Island (1999 and 2007) and Discovery Bay (1999) with mean total length ranging from 25.9 to 35.3 mm. The cohort size range shows good agreement to local age-size observations (Liu 1994b). To avoid mixing between cohorts, only specimens within an average cohort total length ±1 SD were selected for subsequent analysis (Table 1).

Genetic diversity

The five microsatellite loci showed high polymorphism across all the samples, with mean allelic richness (R) ranging from 14.56 to 16.85, with expected heterozygosity (H_E) from 0.884 to 0.922 and observed heterozygosity (H_O) from 0.846 to 0.901 (Table 1). Significant linkage disequilibrium was detected in only one out of 170 tests for individual samples and pair of loci (between loci C583 and C584 in sample L99, $P = 0.022$), but the global test was not significant for all loci pairs across all samples.

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Table 1 *Cellana grata*: allelic richness (R), expected (H_E) and observed (H_O) heterozygosity, inbreeding coefficient (F_{IS}) for each sample and locus, and F_{IS} for pooling of consecutive cohorts (Pooled F_{IS}) following David et al. (1997)

Shore	Cohort	N	Sample code	Parameter	Locus					
					A8	B97	C186	C583	C584	Multilocus
Tiu Chung Chau	1998	22	T98	R	17.06	13.15	13.29	13.74	11.15	13.68
				H_E	0.934	0.910	0.901	0.916	0.874	0.907
				H_O	0.864	0.909	0.773	0.900	1.000	0.889
				F_{IS}	+0.098	+0.024	+0.165	+0.043*	−0.121	+0.043*
	1999	20	T99	R	15.57	14.01	11.07	15.39	10.26	13.26
				H_E	0.918	0.895	0.889	0.923	0.880	0.901
				H_O	0.842	0.850	0.800	0.950	0.842	0.857
				F_{IS}	+0.110	+0.076	+0.125	−0.004	+0.069	+0.075
			T98/T99	Pooled F_{IS}						+0.058*
	2006	31	T06	R	15.27	11.88	14.93	15.84	10.60	13.70
				H_E	0.900	0.896	0.925	0.925	0.871	0.903
				H_O	0.800	0.800	0.850	0.950	0.950	0.870
				F_{IS}	+0.136	+0.133	+0.107*	−0.001	−0.065	+0.063
	2007	30	T07	R	17.30	14.96	12.71	16.60	10.78	14.47
				H_E	0.940	0.925	0.904	0.934	0.853	0.911
				H_O	0.850	0.850	0.900	0.950	0.850	0.880
				F_{IS}	+0.121	+0.107	+0.030	+0.007	+0.029	+0.059
			T06/T07	Pooled F_{IS}						+0.060
Big wave Bay	1998	20	B98	R	14.92	13.39	13.57	15.46	9.30	13.33
				H_E	0.911	0.914	0.913	0.928	0.873	0.908
				H_O	0.900	0.900	0.900	0.900	0.900	0.900
				F_{IS}	+0.038	+0.041	+0.039	+0.055	−0.006	+0.034
	1999	21	B99	R	17.82	14.02	13.58	15.41	11.52	14.47
				H_E	0.940	0.918	0.912	0.909	0.873	0.910
				H_O	0.952	0.905	0.905	0.905	0.905	0.914
				F_{IS}	+0.011	+0.039	+0.032	+0.029	−0.012	+0.020
			B98/B99	Pooled F_{IS}						+0.025
	2006	28	B06	R	15.98	13.02	14.51	15.53	13.18	14.44
				H_E	0.911	0.918	0.918	0.908	0.910	0.913
				H_O	0.900	0.850	0.950	0.950	1.00	0.930
				F_{IS}	+0.038	+0.099	−0.010	−0.021	−0.073	+0.007
	2007	32	B07	R	14.92	13.66	13.53	17.01	10.87	14.00
				H_E	0.924	0.920	0.901	0.930	0.880	0.911
				H_O	0.947	0.850	1.000	0.950	0.950	0.939
				F_{IS}	+0.051	+0.102	−0.084	+0.004	−0.054	+0.005
			B06/B07	Pooled F_{IS}						+0.005
Beaufort Island	1998	20	L98	R	14.94	14.29	14.02	14.43	11.71	13.88
				H_E	0.914	0.923	0.915	0.906	0.890	0.910
				H_O	0.850	0.850	0.850	0.800	0.800	0.830
				F_{IS}	+0.095	+0.104	+0.097	+0.142	+0.126*	+0.113*
	1999	18	L99	R	17.70	13.33	12.99	11.92	12.73	13.73
				H_E	0.920	0.898	0.914	0.888	0.897	0.903
				H_O	0.889	0.833	0.833	0.778	0.944	0.855
				F_{IS}	+0.062	+0.101	+0.116	+0.150	−0.025	+0.081
			L98/L99	Pooled F_{IS}						+0.098*

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Table 1 continued

Shore	Cohort	N	Sample code	Parameter	Locus					
					A8	B97	C186	C583	C584	Multilocus
	2006	33	L06	R	15.41	11.66	16.55	16.70	14.02	14.87
				H_E	0.914	0.888	0.938	0.926	0.916	0.916
				H_O	0.850	0.850	0.900	0.950	1.00	0.910
				F_{IS}	+0.095	+0.068	+0.066	+0.001	−0.066	+0.032
	2007	32	L07	R	19.44	15.75	15.75	15.59	9.96	15.30
				H_E	0.948	0.928	0.928	0.914	0.868	0.917
				H_O	0.850	0.850	1.000	0.950	0.900	0.910
				F_{IS}	+0.128*	+0.109	−0.053	−0.014	−0.012	+0.033
			L06/L07	Pooled F_{IS}						+0.033
Discovery Bay	1998	20	D98	R	15.40	12.32	16.72	13.30	9.49	13.45
				H_E	0.935	0.900	0.930	0.888	0.873	0.905
				H_O	0.900	0.750	0.950	0.750	0.900	0.850
				F_{IS}	+0.105	+0.191	+0.004	+0.180	−0.006	+0.095
	1999	15	D99	R	16.39	13.66	13.00	18.25	10.73	14.41
				H_E	0.920	0.916	0.903	0.933	0.873	0.909
				H_O	0.867	0.867	0.929	0.867	0.867	0.879
				F_{IS}	+0.092	+0.088	+0.009	+0.106	+0.042	+0.068
			D98/D99	Pooled F_{IS}						+0.082
	2006	31	D06	R	16.70	14.64	14.39	14.20	10.89	14.16
				H_E	0.903	0.914	0.910	0.911	0.889	0.905
				H_O	0.800	0.850	0.900	0.900	0.800	0.850
				F_{IS}	+0.139	+0.095	+0.037	+0.038	+0.125	+0.087
	2007	35	D07	R	16.93	13.52	14.70	15.18	12.84	14.63
				H_E	0.923	0.896	0.916	0.918	0.908	0.912
				H_O	0.800	0.950	0.950	0.900	0.900	0.900
				F_{IS}	+0.158	−0.034	−0.011	+0.045	+0.034	+0.039
			D06/D07	Pooled F_{IS}						+0.061*
		408		Total R	16.71	14.23	14.25	15.41	11.42	
				Mean H_E	0.921	0.907	0.913	0.916	0.883	
				Mean H_O	0.859	0.857	0.899	0.897	0.907	

Asterisks indicate deviation from Hardy–Weinberg equilibrium by exact test (* $P < 0.05$)

Deviation from the Hardy–Weinberg equilibrium was observed in only 4 out of 80 cases involving 4 loci ($P < 0.05$; Table 1), and all cases were due to heterozygote deficit with F_{IS} ranging from 0.043 to 0.128. Multilocus estimates of F_{IS} were significant in 2 of the 16 samples (T98 and L98, $P < 0.05$). MICRO-CHECKER results suggested the significant F_{IS} in T98 was caused by the presence of null alleles in locus C583. Genotypes were adjusted for the sample following the Brookfield (1996) null allele estimator 1 method. The subsequent allele frequency-based analyses (F_{ST}, AMOVA and assignment test) did not, however, show any differences before and after the adjustment (data not shown), and so all analyses were performed using the unadjusted data set. For sample L98, MICRO-CHECKER did not reveal the presence of null alleles, suggesting that the deviations from the Hardy–Weinberg equilibrium may be caused by other biological factors. Values of F_{IS} were positive in 60 out of 80 tests (Table 1), indicating a general heterozygote deficit in the samples. Negative F_{IS} were recorded in 19 cases, which showed a bias toward the locus C584 (10 cases) and samples from 2006/2007 collection (13 cases), but none of the estimates was significant. Contrasting F_{IS} were observed in some consecutive cohorts that may represent temporal heterogeneity, so samples were pooled from the consecutive cohorts for F_{IS} computation following David et al. (1997). Significant deviation was evidenced in three of the eight pooled cohorts, namely, T98/T99, L98/L99 and D06/D07 (Table 1), suggesting the existence of localized temporal variation in some shores.

Table 2 *Cellana grata*: across-shore fixation index (overall F_{ST}) and the associated significance (P), inbreeding coefficient (F_{IS}) and the associated significance (P), expected (H_E) and observed (H_O) heterozygosity, and allelic richness (R) for each locus in the two temporal collections

Locus	Overall F_{ST}	P	F_{IS}	P	H_E	H_O	R
1998/1999 collection							
A8	0.0028	0.1105	0.076	0.0413	0.924	0.883	16.23
B97	0.0026	0.0885	0.081	0.1514	0.909	0.858	13.52
C186	0.0021	0.1153	0.076	0.2176	0.910	0.868	13.53
C583	0.0015	0.0493	0.085	0.0114	0.911	0.856	14.74
C584	0.0024	0.2914	0.006	0.5799	0.879	0.895	10.86
All	0.0023	0.0145	0.065	0.0067			
2006/2007 collection							
A8	0.0008	0.2120	0.108	0.0126	0.920	0.850	16.49
B97	0.0006	0.1151	0.085	0.1795	0.911	0.856	13.64
C186	0.0008	0.5215	0.010	0.4750	0.918	0.931	14.63
C583	0.0013	0.7210	0.007	0.8815	0.920	0.938	15.83
C584	0.0004	0.2785	−0.010	0.7820	0.887	0.919	11.64
All	0.0008	0.2891	0.041	0.1673			

A multilocus significant deviation from the Hardy–Weinberg equilibrium was recorded for the 1998/1999 collection ($F_{IS} = 0.065$, exact test: $P = 0.0067$), reflecting overall heterozygote deficiency (Table 2). The exact test for the 2006/2007 collection was, however, not significant ($F_{IS} = 0.041$, exact test: $P = 0.1673$). Three of the five microsatellite markers exhibited higher F_{IS} in the 1998/1999 than 2006/2007 collection, in which only locus C583 showed a change in significance between the two collections. For individual loci, significant deviations were detected in locus A8 and C583 in the 1998/1999 collection and locus A8 only in the 2006/2007 collection (Table 2). Repeated analyses of the data with the exclusion of locus A8 or C583 did not reveal a difference in significance in both temporal collections (A8 excluded: 1998/1999, $P = 0.0214$; 2006/2007, $P = 0.6649$; C383 excluded: 1998/1999, $P = 0.0406$; 2006/2007, $P = 0.0927$).

Multilocus estimates of allelic richness were higher in the 2006/2007 than the 1998/1999 collection (Student's *t*-test: $P = 0.034$). As the change in allelic richness suggested a potential recent change in effective population size, evidence for a recent population bottleneck event was tested. Under the IAM, which is the least conservative model, a significant bottleneck ($H_e > H_{eq}$: $P < 0.05$) was detected in four samples (T98, B98, D99 and B07). These results, however, should be regarded with caution because of the poor correspondence of the IAM to microsatellite evolution (Weetman et al. 2006). Under the most conservative mutation model (SMM) and the more realistic mutation model (TPM) for microsatellites (Di Rienzo et al. 1994), no sign of a bottleneck was evidenced in all the sixteen samples. When the four spatial samples from each cohort were combined for overall analysis, only one significant result for the 1998 cohort under the IAM model was detected. Tests for the 1998 cohort under SMM and TPM models, as well as for the other three cohorts under all the three models, were not significant ($P > 0.05$).

Spatio-temporal genetic differentiation

Genetic differentiation in the two temporal collections was low, but variability in the 1998/1999 collection was higher, with non-overlapping C.I. ($F_{ST} = 0.0023$, 95% C.I. = 0.0014–0.0034) when compared with the 2006/2007 collection ($F_{ST} = 0.0008$, 95% C.I. = 0.0002–0.0013, Table 2). Of the five markers, only locus C583 ($P < 0.0493$) exhibited a significant F_{ST} value. Pairwise genetic differentiation between samples was detected in a few cases using both pairwise F_{ST} and exact tests (Table 3). Pairs showing significant differentiation did not exhibit apparent spatial or temporal patterns, but differentiation was generally more common within the 1998 cohort, and between 1998 and the other cohorts. No significant differentiation was observed in any pair within and between the 2006 and 2007 cohorts, suggesting a homogeneous structure among these cohorts.

Pairwise D_{CE} among the 16 samples revealed weak genetic structuring, but temporal or spatial samples were not well clustered (Fig. 2). Groupings were restricted to selected sample pairs, either from the same cohort of different shores (T99 and L99, B99 and D99) or from different cohorts of the same shore (B99 and B07, T06 and T98). The spatial AMOVA, however, showed a significant genetic structure among shores in the 1998/1999 collection ($\Phi_{CT} = 0.0031$, $P = 0.048$; Table 4). In the 2006/2007 collection, variation among shores was lower and not significant ($\Phi_{CT} = 0.0018$, $P = 0.122$). The variation among samples within shores was close to zero and not significant for both 1998/1999 ($\Phi_{SC} = 0.0005$, $P = 0.703$) and 2006/2007 ($\Phi_{SC} = -0.0006$, $P = 0.785$) collections. The Mantel test exhibited no significant correlation between linearized F_{ST} and hydrographic distances in either 1998/1999 ($r = -0.109$, $P > 0.05$) or 2006/2007 ($r = 0.246$, $P > 0.05$) collections. Partitioning of genetic variability between temporal collections revealed a non-significant structuring between the 1998/1999 and the 2006/2007 collections ($\Phi_{CT} = 0.0010$, $P = 0.055$; Table 5). As a higher differentiation was detected in the 1998/1999 collection, a further AMOVA was carried out for the partition between the 1998 and 1999 samples that revealed no significant differentiation ($\Phi_{CT} = 0.0007$, $P = 0.310$) between the two cohorts.

The N_e estimates using Waples (1989) method for all temporal samples combined revealed a median of 599

Table 3 *Cellana grata*: pairwise F_{ST} (Weir and Cockerham's θ) (above diagonal) and exact tests of genetic differentiation (below diagonal) across the 16 samples

Sample	T98	B98	L98	D98	T99	B99	L99	D99	T06	B06	L06	D06	T07	B07	L07	D07
T98	–	0.0045	0.0061	0.0018	0.0025	0.0035	0.0103*	−0.0003	0.005	0.0089*	0.0027	0.0050	0.0044	0.0134*	0.0063	0.0063
B98	0.1171		0.0043	0.0026	0.0055	−0.0018	−0.0007	−0.007	−0.0043	0.0034	−0.0003	0.0004	0.0016	0.0031	−0.0022	−0.0006
L98	**0.0456**	**0.0397**		0.0097*	0.0041	0.0041	0.0013	−0.0039	0.0018	0.0048	0.0028	0.0037	0.0020	0.0028	0.0083	0.0044
D98	0.3926	0.2394	**0.0076**		0.0095	0.0043	0.0082	−0.0034	−0.0002	0.0123*	0.0035	−0.002	0.0053	0.0060	0.0053	0.0025
T99	0.3438	**0.0403**	**0.0262**	**0.0112**		0.0064	−0.0044	−0.0043	0.0069	0.0043	−0.0014	−0.0015	−0.005	0.0075	0.0070	0.0062
B99	0.3685	0.5966	0.0792	0.2144	0.1042		0.006	−0.0094	0.0016	0.0074	0.0092*	0.0040	0.0015	0.0003	0.0005	0.0037
L99	**0.0192**	0.3274	0.1387	**0.0257**	0.7137	0.1053		−0.0053	0.0029	0.0035	−0.0075	0.0009	−0.0016	0.007	0.0027	−0.0002
D99	0.5122	0.8470	0.5064	0.7153	0.6349	0.9708	0.7233		−0.0041	0.0042	−0.0002	−0.0034	−0.0030	−0.0026	−0.007	−0.0020
T06	0.2107	0.7274	0.0898	0.4508	**0.0357**	0.3649	0.2442	0.6232		0.0006	−0.0013	−0.0046	−0.0001	0.0017	0.0004	0.0002
B06	0.0612	0.2440	**0.0280**	**0.0056**	0.1929	0.1658	0.1739	0.1874	0.5831		0.0008	0.0057	0.0004	−0.0009	−0.0002	0.0078
L06	0.3401	0.3091	0.2432	0.0898	0.4914	**0.0344**	0.9533	0.4550	0.5088	0.3747		0.0016	−0.0029	0.0055	0.0008	−0.0042
D06	0.0933	0.3153	0.0900	0.5231	0.5114	0.1690	0.2265	0.4205	0.8578	0.1545	0.4460		0.0023	0.0068	0.0033	−0.0019
T07	0.3347	0.4563	0.1405	0.1126	0.8985	0.5352	0.5921	0.7890	0.5037	0.5196	0.7249	0.5837		0.0002	−0.0070	−0.0006
B07	**0.0012**	0.1367	0.0573	0.1061	**0.0448**	0.6445	**0.0312**	0.5424	0.1687	0.6210	0.0568	0.0726	0.3036		−0.0010	0.0079
L07	0.1051	0.7699	**0.0074**	0.0688	0.1618	0.5481	0.1251	0.9541	0.5147	0.7585	0.6239	0.2776	0.9638	0.2265		0.0052
D07	0.1564	0.5011	**0.0346**	0.1240	0.0522	0.3638	0.3819	0.6909	0.4946	0.0833	0.8826	0.6628	0.8455	0.0563	0.1955	–

Significance of θ was tested with 10,000 permutations (* $P < 0.05$). Exact tests with P values < 0.05 were indicated in bold, all values were not significant after sequential Bonferroni correction. Sample codes as in Table 3

with 95% confidence limits (CL) of 352–11,397. Assignment tests revealed a mean value of only 8% (ranged from 0 to 23.8%) of individuals that was correctly assigned to their original samples (Table 5). The proportion of assignment to the same shores was 28.3% (10.6–47.7%), and for assignment to the same cohorts was 33.6% (14.3–52.6%). The 1998/1999 collections showed a higher rate of correct assignment to the original sample (ANOVA, $P = 0.018$) and the rate of assignment to the same shore ($P = 0.043$) than the 2006/2007 collection. In contrast, the rates for assignment to the same cohort ($P = 0.794$) and wrong assignment ($P = 0.581$) were not significant.

Discussion

Analysis of temporal samples of *Cellana grata* over a 10 year period reveals minor, patchy genetic structure without clear spatial or temporal patterns. The major observation is the contrasting genetic variability between collections separated by 10 years (as indicated by the significant heterozygote deficiency from Hardy–Weinberg equilibrium, the higher global F_{ST} and significant spatial AMOVA in the 1998/1999 but not in the 2006/2007 collection). The genetic partitioning between the two temporal collections was, however, weak. Such genetic heterogeneity over such small geographic and temporal scales is generally believed to result from temporal variation in the genetic composition of recruits (Johnson and Black 1982; Li and Hedgecock 1998; Selkoe et al. 2006).

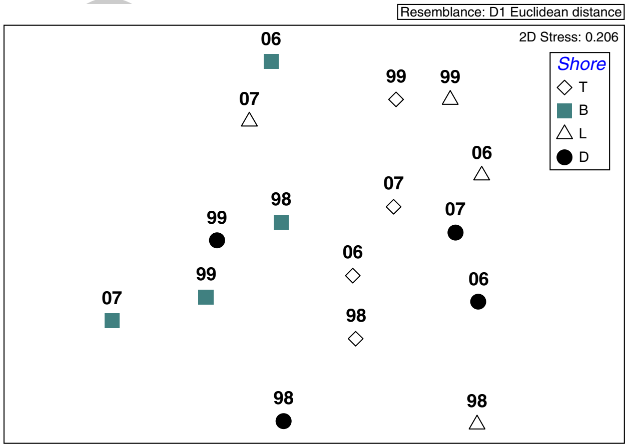

Fig. 2 *Cellana grata*. nMDS of pairwise sample by sample Cavalli-Sforza and Edwards chord distance

Heterozygote deficiency

Heterozygote deficiencies relative to Hardy–Weinberg expectations (positive F_{IS}) were estimated for selected loci and overall for the 1998/1999 collection. Such deficiency is not uncommon among Patellogastropods (Corte-Real et al. 1996; Weber and Hawkins 2006; de Aranzamendi et al. 2008). The observed heterozygote deficiencies may be due to the presence of null alleles or biological processes such as inbreeding and Wahlund effects. In this study, it is unlikely for the null or non-amplifying allele to cause the observed heterozygote deficiencies as the level of F_{IS} changed between the temporal collections for the same loci. This is especially true as it is using the corrected allele

Table 4 *Cellana grata*: AMOVA for spatial population genetic structure in 1998/1999 and 2006/2007 collections, and temporal population structure between the two collections

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*
Four shores in 1998/1999						
Among shores	3	8.79	0.00727 Va	0.31	$\Phi_{CT} = 0.0031$	**0.048**
Among samples within shores	4	9.46	0.00106 Vb	0.05	$\Phi_{SC} = 0.0005$	0.703
Within samples	304	702.32	2.32556 Vc	99.64	$\Phi_{ST} = 0.0036$	0.223
Four shores in 2006/2007						
Among shores	3	7.834	0.00413 Va	0.18	$\Phi_{CT} = 0.0018$	0.122
Among samples within shores	4	9.125	−0.00137 Vb	−0.06	$\Phi_{SC} = -0.0006$	0.785
Within samples	496	728.825	2.33598 Vc	99.88	$\Phi_{ST} = 0.0012$	0.538
1998/1999 versus 2006/2007						
Among the temporal collections	1	3.245	0.00231 Va	0.10	$\Phi_{CT} = 0.0010$	0.055
Among samples within collections	14	35.209	0.00468 Vb	0.20	$\Phi_{SC} = 0.0020$	0.301
Within samples	815	1,413.143	2.33086 Vc	99.70	$\Phi_{ST} = 0.0029$	0.161

Table 5 *Cellana grata*: assignment tests to determine the likelihood of an individual being classified in their original population by their genotypes conducted using GENECLASS2

Sample	Assigned to correct shore and cohort		Assigned to same shore		Assigned to same cohort		Assigned to wrong shores and cohorts	
	%	95% C.I.	%	95% C.I.	%	95% C.I.	%	95% C.I.
T98	4.8	13.6 ± 16.3	38.1	41.3 ± 12.5	28.6	28.4 ± 18.2	38.1	44.0 ± 7.1
B98	21.1		31.6		42.2		47.4	
L98	23.8		47.6		28.6		47.6	
D98	4.8		47.7		14.3		42.9	
T99	11.1	9.7 ± 3.0	33.3	26.8 ± 12.1	44.4	37.1 ± 12.6	33.3	45.8 ± 16.2
B99	9.5		19.0		33.3		57.1	
L99	11.1		33.3		27.8		50.0	
D99	7.1		21.4		42.8		42.9	
T06	0.0	2.6 ± 4.8	21.1	18.0 ± 8.0	21.1	33.4 ± 14.2	57.9	51.3 ± 7.9
B06	0.0		19.0		33.3		47.6	
L06	5.3		21.1		36.9		47.4	
D06	5.3		10.6		42.1		52.6	
T07	0.0	6.2 ± 7.7	21.1	27.2 ± 21.8	52.6	34.5 ± 25.16	26.3	43.5 ± 19.7
B07	4.8		47.7		14.3		42.9	
L07	10.5		21.0		36.8		52.6	
D07	9.5		19.0		38.1		52.4	
Total	8.0 ± 3.6		28.3 ± 6.3		33.6 ± 5.7		46.3 ± 4.5	

Sample codes as in Table 1

frequency as suggested by MICRO-CHECKER for locus C583 revealed no change in the significance of the Hardy–Weinberg equilibrium, F_{ST} or AMOVA. Inbreeding or localized recruitment due to the restricted dispersal of gametes or larvae has been hypothesized to cause heterozygote deficiencies (Magalon et al. 2005; Costantini et al. 2007). Such phenomenon is, however, more common in species with low dispersal ability (Ben-Shlomo et al. 2001). *Cellana* limpets are broadcast spawners with a high dispersal potential (Bird et al. 2007). The dispersal ability of this species is also supported in this study, at least over the sampling range, by the panmictic structure among spatial samples in the 2006/2007 collection. In addition, heterozygote deficiencies were not congruent among loci in the data, indicating inbreeding is unlikely.

The Wahlund effect, reduction in heterozygosity resulting from sampling across subdivided populations, has been suggested in different taxa for cases of elevated F_{IS} (David et al. 1997; Raymond et al. 1997). Johnson and Black (1984b) reported a patchy genetic structure with general deficit of heterozygotes in *Siphonaria* limpet species from the Australian coast and proposed that the deficiency was caused by mixing of larvae from different sources over a small geographic scale. On the other hand, mixing of subdivided populations could happen temporally, such as surges of larval within and between spawning events may be contributed from genetically distinct populations (Lenfant and Planes 2002; Maes et al. 2006). In this study, the observed heterozygosity reflected the potential Wahlund effect in the collections. A characteristic evolution of the deficit of heterozygotes under a temporal Wahlund effect is an increase in heterozygote deficiency in relation to the increasing number of cohorts pooled together (David et al. 1997). Some of the samples showed significant heterozygote deficiency when pooling consecutive cohorts, particularly for sample D06 and D07 that did not deviate before pooling, suggesting a potential temporal Wahlund effect. However, the low heterogeneity among the samples did not support the presence of genetically distinct source populations. This less profound structuring may be the result of recruitment variation at a localized scale in which different portions of the source population are recruited to each site depending on the localized oceanographic or environmental conditions at that time. Additionally, such uneven genetic distribution is likely to vary from year to year and lead to the contrasting genetic structure observed between the temporal collections in this study.

Spatio-temporal genetic differentiation

There was a higher degree of genetic differentiation in the 1998/1999 collection when compared to the 2006/2007 collection. The spatial AMOVA indicated that this partitioning was contributed mainly by the variation among shores; assignment tests also revealed higher assignment rate to shores in the 1998/1999 collection. The lack of IBD, however, suggests that the distance between shores is not the determining factor for this variability. Such unpatterned spatial differentiation may reflect a random and uneven genetic composition among the shores. In other studies with comparable geographic ranges, spatial genetic structure of intertidal nudibranchs has been related to the scale of larval dispersal of specific years (Lambert et al. 2003), while Pedersen et al. (2000) also identified genetic heterogeneity within and between cohorts of mussel settlers. Both these studies regarded these patterns to be a result of large variation in recruit composition among cohorts. In the marine environment, reproductive success of free spawning adults is largely influenced by the variable oceanic conditions (Underwood and Fairweather 1989; Gaines and Bertness 1992) that may operate at various scales at different stages of the spawning process, affecting fertilization dynamics (Hodgson et al. 2007), larval dispersal and/or retention (Ayata et al. 2009) and survivorship (Botsford et al. 1994). The survivorship of progeny is unpredictable, and successful recruits may be contributed by only a small portion of the adult population in each spawning event. The extent of how many and which individuals are successful, therefore, may be largely determined by chance. Li and Hedgecock (1998) hypothesized that genetic heterogeneity among cohorts should be detectable if only a small proportion of adults are contributing to the recruits. It is noteworthy that significant pairwise genetic differentiation was more common within the 1998 cohort and between 1998 and the other cohorts. High fluctuation in recruitment over cohorts/spawning events has been proposed to boost genetic drift, decrease effective population size and cause temporal genetic structuring (Lenfant and Planes 2002; Maes et al. 2006). The differentiation between the 1998/1999 and the more homogeneous 2006/2007 collection was, however, non-significant (although $P = 0.055$). Firstly, these observations imply a generally consistent larval supply over time and secondly that genetic drift caused by the spatial differentiation was not high enough to generate a substantial temporal structure.

It is surprising that the estimates of effective population size were not high over the study period, although the confidence limits were wide, and so this conclusion should be viewed with caution. Similar estimates were observed for oysters from Chesapeake Bay (Rose et al. 2006), and the low ratio of N_e to the population size was attributed to high variation in reproductive success. Lee and Boulding (2007) showed that genetic structure of *Littorina keenae* in the northeastern Pacific was temporally stable in general ($N_e \approx 30{,}000$), but low N_e (135) at selected sites reflected a 'sweepstake recruitment'. A review of effective to actual population size (N_e/N) over 102 taxa suggested an average of only 0.10–0.11 of the population contributes progeny (Frankham 1995), while Turner et al. (2002) found that the N_e was five to three orders below the adult census size. The actual population sizes were not measured for each shore in this study. An abundance survey along various shores spanning the Hong Kong coastline in 1998–1999 revealed an average of 9.01 *Cellana grata* m^{-2} (SD = 44.06, $n = 45$). Assuming even population density over the survey area (3 m wide × 300 m along coastline × 4 shores), the range of N_e/N estimates would be $(352 \text{ to } 11{,}397)/(9.01 \times 3 \times 300 \times 4) = 0.011\text{–}0.351$. Such values should, however, be interpreted with care due to the involvement of uncertain parameters such as the juvenile/adult proportion, sex ratio. The order of the estimates, however, does indicate the potentially small portion of effective spawners in the population, and hence potential susceptibility to recruitment variation.

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The arguments for recruitment variation generally highlight the importance of pre-settlement factors governing the resultant genetic differentiation (Hedgecock 1994; Flowers et al. 2002), although microenvironmental post-settlement selection has also been proposed (Johannesson et al. 1995). *Cellana grata* suffers from regular on-shore mass mortality during summer, due to heat and desiccation stress, where up to 80% of the population can be killed (Williams and Morritt 1995; Ngan 2006). A vast reduction in the effective population size may lead to a population bottleneck and results in reduction in allele number and gene diversity (Cornuet and Luikart 1996). Despite this potential, analyses did not reveal strong proof of a bottleneck under the more conservative and realistic SMM and TPM models (Di Rienzo et al. 1994). The lack of detection of a bottleneck implies the actual spawning population is either not affected or maintained at a relatively constant level by the summer mass mortality.

Although *Cellana grata* populations showed some degree of fine scale recruitment variation, the resulting genetic drift is probably not robust enough to have a strong impact on their overall genetic structure. Significant differentiation would be expected if the recruitment variation acted at a persistent and larger scale, but this was only found within the 1998/1999 samples and was not recorded in the 2006/2007 collection or between the two temporal collections. This agrees with the findings of Selkoe et al. (2006) who performed extensive analysis to reveal the combined effect of family structure (existence of siblings) and changes in larval delivery on the fine scale genetic structure in cohorts of kelp bass populations. As in the present study, Selkoe et al. (2006) concluded that the degree of genetic drift was not strong enough to impact the adult genetic structure. The potential driving forces affecting genetic population structure of limpets in this study are unlikely to operate in a consistent pattern over time, and any genetic variance was probably counteracted by gene flow among the shores. While the degree of the detected genetic differentiation only amounts to slight differences in allele frequencies that may not generate substantial biological effects (Waples 1998; Hedrick 1999), the results suggest the phenomenon of potential fine scale structuring due to recruitment variation under a scenario of broad scale genetic homogeneity. This work therefore highlights the interacting effects between recruitment dynamics and population connectivity in determining intertidal population genetic structure.

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