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Genetic evidence for kin aggregation in the intertidal acorn barnacle (*Semibalanus balanoides*)

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Abstract

It is generally assumed that larvae of benthic species are thoroughly mixed in the plankton and distributed randomly at settlement. Yet, it has also been hypothesized that a combination of larval gregarious behaviour coupled with particular oceanographic conditions may prevent larvae from mixing completely, and result in nonrandom spatial distributions following settlement. Using microsatellite markers, the main objective of this study was to investigate the occurrence of statistical connections between relatedness and settlement in the intertidal acorn barnacle from the Gulf of St Lawrence, Canada. A second objective was to test the hypothesis that patches of kin-related individuals came from a common parental site. Our results indicated that a significant number of barnacles within a given sample were more closely related than expected by chance despite the enormous potential for admixture during the planktonic phase. Thus, eight out of 37 samples analysed had relatedness values significantly higher than expected from random settlement. Moreover, analyses of sibship network construction and network complexity tests provided evidence for the occurrence of networks within several samples that were characterized by strong connections among individuals. Thus, nonrandom planktonic dispersal associated with relatively stable oceanic currents, as well as additional ecological factors to be rigorously investigated (e.g. behavioural mechanisms), may be more important in determining patterns of genetic structure in marine benthic invertebrates than generally assumed. Therefore, documenting genetic patterns associated with kin aggregation should be a fruitful and an important avenue for future studies in marine invertebrates.

Keywords: family structure, heterozygote deficit, kin aggregation, marine invertebrates, network analysis, relatedness

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Introduction

Marine benthic invertebrates are often characterized by separate benthic (sessile) and planktonic (larval) phases linked by a settlement event during their life history (Thorson 1950). Because the mobility of such species is severely limited at the adult stage, events incurred during their planktonic stage are determinant for their geographical range, genetic structure, and ultimately their evolutionary history (Johnson *et al*. 2001; Riginos & Victor 2001; Underwood & Keough 2001; Kinlan & Gaines 2003; Taylor & Hellberg 2003). The capacity to disperse at the planktonic stage is in turn dependent on the duration of larval life, larval behaviour and hydrographic regimes (Le Fèvre & Bourget 1992; Jenkins & Hawkins 2003). However, evaluating these effects has been largely inferential due to logistical constraints for direct larval tracking (Levin *et al*. 1993; Eckert 2003; Jenkins & Hawkins 2003; Shanks *et al*. 2003).

It has been widely assumed that during the planktonic period, larvae originating from different locations and/or parents are completely free to mix (e.g. Le Fèvre & Bourget 1992). Yet, there is evidence showing that discrete patches of larvae could be maintained in the water column on a scale of days to weeks (Kordos & Burton 1993; Natunewicz & Epifanio 2001; Natunewicz *et al*. 2001). This raises the

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possibility that larvae may sometimes disperse as nonrandom groups without complete admixture, and that under certain conditions, larvae from the same family group (i.e. linked by half-sib or full-sib relationships) could be transported in the same water mass and settle in close proximity, thereby influencing the genetic structuring at the adult stage. Thus, many organisms with high dispersal rates show much greater spatial heterogeneity over short distances than would be expected from their dispersal potential, resulting in high heterozygote deficiencies (e.g. Zouros & Foltz 1984; Watts *et al*. 1990; Lehmann *et al*. 2003; Smith *et al*. 2004) and disparate genetic patterns resulting in 'chaotic patchiness' (e.g. limpets, Johnson & Black 1982, 1984; bivalves, Gosling & Wilkins 1985; David *et al*. 1997; Huvet *et al*. 2000; echinodermata, Nishida & Lucas 1988; Watts *et al*. 1990; Moberg & Burton 2000; Addison & Hart 2004).

Using microsatellite markers, the main objective of this study was to investigate the occurrence of statistical connections between relatedness and settlement in the acorn barnacle from the Gulf of St Lawrence, Canada. A second objective was to test the hypothesis that each patch of highly related specimens came from a common parental site, thus producing a short-lived, random, family link between two sites. The acorn barnacle is one of the most abundant benthic species of the intertidal zone (Barnes & Barnes 1976). Several biological and ecological features could make the acorn barnacle particularly prone to show patterns of kin aggregation, including their gregariousness (Knight-Jones & Stevensson 1950; Knight-Jones 1953; Head *et al*. 2004) and their capacity to respond to chemical inducers derived from conspecific adults and larvae (Knight-Jones 1953; Crisp & Meadows 1962; Yule & Walker 1985; Clarke *et al*. 1994; Miron *et al*. 1996; Berntsson *et al*. 2004). Moreover, it reproduces internally with one or several neighbours (Anderson 1994) and consequently, thousands of larvae representing reproductive aggregations composed of full- and half-sibs progeny may be simultaneously released. Finally, oceanographic conditions where the species is found may be conducive to limiting larval mixing despite high dispersal potential (Dufresne *et al*. 2002; Véliz *et al*. 2004).

Materials and methods

Sampling

A total of 1250 individuals were sampled from the intertidal zone of the Gulf of St Lawrence, Canada (Fig. 1). There, the shore consists of over 200 km of sandy beaches and barnacles are only found on sporadic rocky outcrops of a maximal dimension of 300 m in length (Brind'Amour *et al*. 2002). Two cohorts (2000 and 2001) sampled at two time periods were used in this study: June (during larval settlement) and October (4 months after settlement) (Tables 1 and 2). Yearly cohorts are easily distinguishable because massive larval recruitment occurring during a short period (Véliz *et al*. 2004) each year results in nonoverlapping sizes among cohorts. Two and four locations were sampled for cohort 2000 and 2001, respectively (Fig. 1), and between six to 14 samples were obtained for each location in a given year, a sample corresponding to a group of individuals collected within a microhabitat at a given site (see details in Véliz *et al*. 2004). An average of 32 individuals were randomly collected for each sample along approximately 100 m of rocky outcrop and were preserved at −80 °C until genetic analyses.

Microsatellite genotyping

Levels of genetic diversity within and between samples were assessed using variation at five microsatellite loci: two tetranucleotid repeats (3GATA, 4GATA) and three dinucleotide loci (*SEBAL*13, *SEBAL*14 and *SEBAL*35), according to Dufresne *et al*. (1999, 2002). Complete individuals were used for DNA extraction. Tissue digestion and DNA extraction were performed following the MCIA (methylene chloride: isoamyl alcohol) procedure described in Claxton & Boulding (1998). Polymerase chain reaction (PCR) amplification was performed as described in Dufresne *et al*. (1999). Microsatellite fragments were visualized electrophoretically on an automated DNA sequencer (Base Station MJ Research) using 0.16 μL standard Rox 500, 0.12 μL formamide and 0.072 μL loading buffer and 2 μL of diluted PCR product. Microsatellite allele sizes were estimated against the internal size standard using the CARTOGRAPHER software (MJ Research).

Data analysis

Allele frequencies and values were calculated using GENEPOP (version 3.1; Raymond & Rousset 1995). Conformance to Hardy–Weinberg expectation (HWE) of genotypic proportions was tested using the permutation test (5000 iterations) implemented in GENETIX software (Belkhir *et al*. 2000).

Kin aggregation permutation tests

In order to test for possible kin aggregation, the pairwise relatedness r_{xy} statistics was computed by KINSHIP (version 1.2; Queller & Goodnight 1989) for each of the four temporal sampling periods; June and October for 2000 and 2001 (Fig. 1). Based on these four sets of r_{xy} values, a permutation test was performed for each sampling period (100 000 iterations). Briefly, for a given sampling period, r_{xy} values were computed for each pair of individuals provided they came from a same sample, and the sum S0 of these r_{xy} values was computed for each sample and then over all samples. In order to obtain a distribution of *S* value

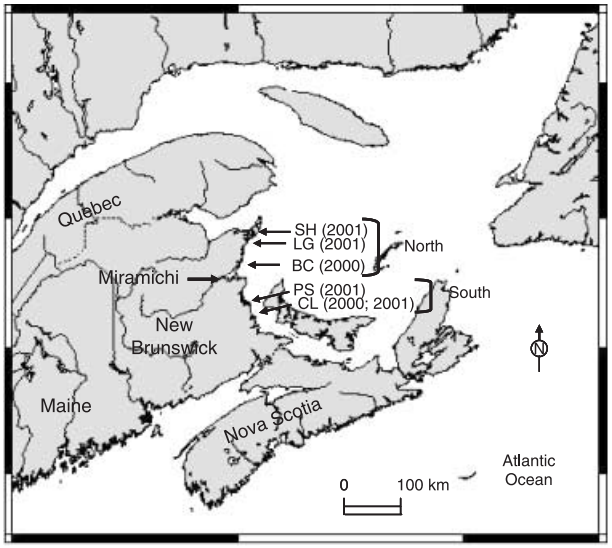

Fig. 1 Sampling sites of *Semibalanus balanoides* located north and south of the Miramichi estuary in the Gulf of St Lawrence. Abbreviations; LG, Le Goulet; SH, Shippagan; BC, Burnt Church; PS, Pointe Sapin; CL, Cap Lumière.

for each sample under H_0 (H_0: random distribution of specimens over samples within a sampling period), the individuals were randomly permuted across samples within a given sampling period and sample S values were computed. For each of the sampling periods, this was done 100 000 times and *P* values were obtained for each sample and also globally over all samples. The permutation procedure was coded in the computer algebra program MAPLE 9.5 (2004) and a detailed description of the algorithm is provided in Appendix 3.

Sibship network construction

Since we were mainly interested to investigate the possible occurrence of kin aggregation soon after settlement, and thus minimizing selective effects (see Véliz *et al*. 2004), this analysis was performed only on June samples of both years. Thus, each sample of June 2000 and June 2001 was partitioned into one or several networks based on an extended notion of sibship. Here, individuals were grouped together when connected through a chain of half- or full-sib intermediate individuals. If one thinks of the half- and full-sib relationships as one compound relationship, then the latter can be characterized as 'sharing at least one parent'. Therefore, within sibship networks, any two members are linked by a chain of parent-sharing connections, thus representing 'extended sibship relationships' (Fig. 2). Note that all pairs of members of a given network may not be full- or half-sibs. The motivation behind the construction of sibship networks is based on the well-documented observation that mating among *Semibalanus balanoides* individuals can only take place when one individual is within penile distance from other potential mates (Anderson 1994). Therefore, all members of a given sibship network must necessarily come from a same reproductive site.

In order to build the sibship networks from a given sample, we first established for any pair of specimens whether they shared at least one parent or not. These results were then collected into a logical matrix where 0 and 1 entries, respectively, meant 'share no parent' and 'share at least one parent', i.e. are either half- or full-sibs. Finally, each logical matrix was translated into sibship networks by using procedures from the graph theory package of MAPLE 9.5 (2004). Note that the latter translation from the logical

Table 1 Cohort 2000: Sample size (n), average relatedness value (r_{xy}), permutation tests for *P* values (100 000 iterations), observed number of connections (NC), number of individuals connected in the main network (NIC) and *P* values (50 000 iterations). Note that NC and NIC were not estimated for October (see Materials and methods). Sample number and habitat description: LS, low sheltered; LE, low exposed; HS, high sheltered; HE, high exposed

June 2000 Location	Burnt Church		Cap Lumière			
Sample	1 (LS)	2 (HS)	3 (LS)	4 (LE)	5 (HS)	6 (HE)
n	29	29	52	57	52	52
r_{xy}	–0.01	–0.02	0.04	0.04	–0.01	–0.01
P	0.638	0.797	0.229	0.005	0.795	0.916
NC	2	1	14	39	2	3
NIC	3	2	11	22	3	4
P	0.740	0.974	0.262	0.005	0.991	0.945
October 2000						
n	25	23	61	76	49	35
r_{xy}	0.01	–0.01	0.01	0.02	0.01	–0.02
P	0.335	0.598	0.389	0.121	0.454	0.884

Table 2 Cohort 2001: Sample size (n), average relatedness value (r_{xy}), permutation tests for P values (100 000 iterations), observed number of connections (NC), number of individuals connected in the main network (NIC) and P values (50 000 iterations). Note that NC and NIC were not estimated for block 4 (see Materials and methods). Sample number and habitat description: LS, low sheltered; LE, low exposed; HS, high sheltered; HE, high exposed

June 2001	Le Goulet		Ship pagan				Pointe Sapin				Cap Lumière			
Location	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sample	(LS)	(HS)	(LS)	(LE)	(HS)	(HE)	(LS)	(LE)	(HS)	(HE)	(LS)	(LE)	(HS)	(HE)
n	31	24	22	20	31	31	31	22	27	32	24	32	30	31
r_{xy}	0.02	0.02	0.02	0.03	0.03	0.00	0.03	−0.01	0.07	−0.00	0.05	0.02	0.01	0.03
P	0.111	0.18 9	0.156	0.178	0.407	0.068	0.159	0.223	0.002	0.517	0.017	0.134	0.280	0.089
NC	9	2	1	0	2	1	5	5	4	3	2	6	2	11
NIC	9	3	2	0	3	2	4	3	4	4	3	5	3	10
P	0.049	0.901	0.901	1.000	0.786	0.990	0.245	0.075	0.252	0.568	0.564	0.186	0.756	0.024

October 2001	Le Goulet		Shippagan				Pointe Sapin				Cap Lumière			
Location	1	2	3	4	5		6		7		8	9	10	11
Sample	(LS)	(HS)	(LS)	(LE)	(HS)	_	(LS)	_	(HS)	_	(LS)	(LE)	(HS)	(HE)
n	32	25	30	28	30		32		29		29	31	26	30
r_{xy}	0.02	0.03	0.10	0.07	0.06		−0.02		0.06		0.07	0.01	0.03	0.03
P	0.235	0.155	0.000	0.005	0.010		0.938		0.007		0.003	0.295	0.129	0.113

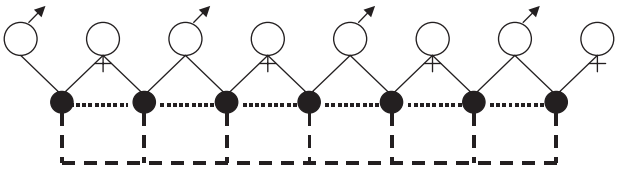

Fig. 2 Schematic representation of the notion of extended sibship relationship. Unfilled disks stand for parents. Black disks are the offspring. Continuous, dotted and dashed lines represent parental, 'sharing at least one parent' and extended sibship relationships, respectively. Note that all pairs of offspring belong to the extended sibship relationship, i.e. are extended sibs although most pairs do not share a parent. Since barnacles are hermaphrodite, individuals identified as males and females could also be the alternate gender.

matrix to sibship networks is completely deterministic. As for the parent-sharing decisions, i.e. the 0 s and 1 s in the logical matrix, they were computed based on a threshold for telling apart unrelated vs. related (half- or full-sibs) individuals. This threshold was established by randomly generating 100 000 pairs of r_{xy} values from unrelated individuals based on the observed allele frequency distributions over all barnacles that were used in this study using KINSHIP (Queller & Goodnight 1989). We considered that type I error was more important than type II error (see Blouin *et al*. 1996), and therefore used a conservative threshold for differentiating unrelated from related barnacles. Thus, taking $\alpha = 0.01$, the threshold between unrelated and related values of relatedness was 0.67. Hence, we scored a 1 for parent sharing when the observed r_{xy} value was larger than 0.67 and a 0 otherwise. Specimens thus composing distinct networks within a given sample may either reflect the existence of truly separate reproductive groups at a same site, the result of incomplete sampling or type II error.

Network complexity tests

A sample comprising unrelated specimens could produce what would appear to be an extended sibship network as a consequence of type I error. Therefore, we devised a simulation procedure in order to test the probability of random formation of networks as complex as the most complex one found within each sample collected in June for both the 2000 and 2001 cohorts. A straightforward means of assessing complexity within a given network is to count the number of connections within that network. Consequently, we defined NC as the number of connections found in the largest sibship network built from a sample, and NIC as the number of individuals comprised in such a network. For each real sample, 50 000 samples of the same size made up of unrelated artificial specimens were randomly generated to obtain a distribution of the associated random variable NC. The observed NC value for each sample within each block was then compared to the NC distribution and a *P* value was computed.

Results

Microsatellite loci were moderately to highly polymorphic, ranging from six alleles at locus *SEBAL*13 to 36 alleles at locus *SEBAL*14 over all individuals analysed. Exact tests revealed significant departures from HWE within samples (Appendices 1 and 2), but deviations were not associated to any specific locus, sampling period or sample (Kendall's concordance coefficient = 0.2410; $P = 0.1481$). Therefore, null alleles do not appear to be responsible for the observed deviations from HWE.

Kin aggregation permutation tests

The permutation test on pairwise r_{xy} values revealed levels of relatedness significantly higher than those expected from a random mixing in several samples (Tables 1 and 2). Thus, eight samples out of 37 showed P values under 0.05, among which six were under 0.01. Average r_{xy} values that were significant varied between 0.04 and 0.10. P values were not significant for all samples collected in 2000 except for sample 4 collected in June ($P = 0.0047$). Thus, global P values were not significant for both June and October 2000. For June 2001, two samples (9 and 11) had significant P values, and three others (1, 6 and 14) showed intermediate values ranging from 0.068 to 0.111. This resulted in a global P value of 0. For October 2001, five samples (3, 4, 5, 7, 8) out of 11 had highly significant P values, which also resulted in a global P value of 0.

Sibship network construction and complexity network test

The analyses of sibship network construction and complexity network tests further supported the evidence for nonrandom kin aggregation in several samples of barnacles following settlement (Tables 1 and 2). Thus, the P value for sample 4 (June 2000) was highly significant at 0.005, and the number of connections, and number of individuals connected within the main network were 39 and 22, respectively. All other samples collected in June 2000 had relatively high P values (0.2618 or higher). For June 2001, two samples (1 and 14) had P values below 0.05. NC and NIC values for these samples were 9, 11, 9 and 100, respectively. Except for sample 8 (0.0748), all other P values were at least as high as 0.1864. The largest networks relating individuals for sample 4 (June 2000) and sample 14 (June 2001) are shown in Fig. 3.

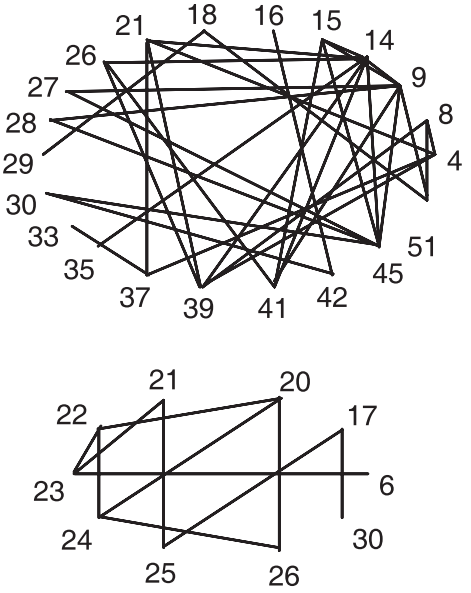

Fig. 3 Top figure: graph of the main network of sample 4 (June 2000). It involves 22 member specimens linked together by 39 connections. Each connection means 'sharing at least one parent' and all pairs of specimens of the network are extended sibs since there is a parental path between any two members of the network. The associated P value (50 000 iterations) was found to be 0.005. Bottom: graph of the main network of sample 14 (June 2001). It comprises 10 specimens and 11 connections. The associated P value (50 000 iterations) was found to be 0.024.

Discussion

Genetic evidence for kin aggregation

The main objective of this study was to test for the occurrence of nonrandom, kin-related settlement in the acorn barnacle. Despite the use of a conservative threshold for telling apart unrelated and related individuals, we observed that eight out of 37 samples analysed showed a level of mean relatedness significantly higher than expected from random settlement. The analyses of sibship network construction and network complexity tests provided further evidence for kin-related settlement by identifying networks of individuals within several of these samples that were connected by kin relationships. These results therefore indicate that a significant number of barnacles within a given sample were more closely related than expected by chance despite the enormous potential for admixture during their planktonic phase.

Several reproductive features make the acorn barnacle particularly prone to show patterns of kin aggregation. First, mating among acorn barnacles can only take place when one individual is within penile distance from other potential mates (Anderson 1994). Moreover, the acorn barnacle is a highly fecund hermaphrodite, each adult producing up to 5000 larvae (Véliz *et al*. 2006a). Barnacles also reproduce internally with one or several neighbours (Anderson 1994), and consequently, thousands of larvae representing reproductive aggregations composed of full- and half-sibs progeny may be simultaneously released. Given these characteristics, barnacles originating from distinct reproduction sites (i.e. rock outcrops) are very unlikely to be related, such that

the most parsimonious explanation for the existence of aggregations of related barnacles is that they originate from parental aggregations that were located within a same reproductive site. The reproductive characteristics of barnacles also increase the probability that offspring from the same site be parentally interconnected even when they do not form a single group of siblings, and as such, may favour the production of large numbers of parentally connected offspring. Yet, such reproductive features are not sufficient to ensure that related individuals remain aggregated until settlement, given their planktonic journey over long distances in the water column.

Additional hypothetical explanations must therefore be invoked for the maintenance of kin-related larval aggregations throughout their planktonic stage. First, oceanographic conditions where the species is found may be conducive to limited larval mixing despite high dispersal potential. Namely, it has been shown that oceanographic conditions may influence dispersal of barnacle larvae and their settlement (Roughgarden *et al*. 1988; Le Fèvre & Bourget 1992; Hansson *et al*. 2003; O'Riordan *et al*. 2004; Hare *et al*. 2005). In the Gulf of St Lawrence in particular, the prevailing strong north–south unidirectional oceanographic currents (Drouin *et al*. 2002; Véliz *et al*. 2006b) could contribute to reducing larval admixture and keeping larvae from the same parental clump together in the water column until settlement, in comparisons with oceanic conditions where eddies and internal waves prevail (Pineda 2000). Second, behavioural mechanisms could also contribute to nonrandom larval settlement. As reported for many barnacle species, kin aggregation at settlement could be reinforced by gregarious behaviours in the acorn barnacle (Knight-Jones & Stevensson 1950; Knight-Jones 1953; Head *et al*. 2004), given their capacity to respond to chemical inducers derived from conspecific adults and larvae (e.g. Knight-Jones 1953; Crisp & Meadows 1962; Rittschof *et al*. 1984; Yule & Walker 1985; Clarke *et al*. 1994; Miron *et al*. 1996; Berntsson *et al*. 2004). Also, cyprid–cyprid interactions have been shown to stimulate settlement in several species of barnacles (Yule & Walker 1985; Clarke *et al*. 1994; Head *et al*. 2004). Assuming that chemical communication could occur during early larval stages, kin recognition during planktonic development could also contribute to maintaining kin-related larvae together until settlement. This, however, remains to be empirically tested.

Possible consequences of related settlement and their implication for genetic population studies

Whereas potential for long-range dispersal is generally expected to result in homogeneous distribution of genetic diversity within a population, including in the acorn barnacle (Dufresne *et al*. 2002), this situation does not always apply to marine benthos (Palumbi 2003). Indeed, many population genetics studies have reported strong heterozygote deficits, as well as disparate patterns of spatial genetic structuring (chaotic patchiness) in nonselfing species with long pelagic larval phases and high potential for dispersal (Palumbi 2003). Explanations for this phenomenon generally fall into four categories, including selective advantage of homozygotes, nondetection of polymorphism due to aneuploidy or null alleles, Wahlund effect and inbreeding (David *et al*. 1997; Sokolov *et al*. 2003). Here, we showed that nonrandom aggregations of related individuals can occur in marine benthic species with long larval phases, which could therefore represent an additional cause for both the deficits in heterozygotes and small-scale genetic structuring regularly reported in benthic species despite their high potential for dispersal during the larval stages.

To conclude, a combination of particular reproductive characteristics, nonrandom planktonic dispersal associated with relatively stable oceanic currents, as well as additional ecological factors yet to be rigorously investigated (e.g. behavioural mechanisms) may play a more important role in shaping patterns of genetic structure of marine benthic invertebrates than generally assumed. However, it is important to note that such genetic patterns may not represent a true signal of stable population structure, but rather an effect of sampling bias running counter to the implicit assumption of random sampling. Indeed, Allendorf & Phelps (1981) emphasized 25 years ago that fish (namely salmonids) progeny produced by a nonrandom and relatively small effective number of breeders within a given population may result in genetic differences among groups of individuals within a single panmictic population. Our results indicate that the so-called 'Alendorf-Phelps' effect may apply to highly abundant species such as the acorn barnacle. Clearly, documenting the determinants of kin aggregation and its impact on spatial patterns of genetic structuring represents an intriguing research avenue for future studies in marine invertebrates (MacColl *et al*. 2000; Coltman *et al*. 2003).

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This research is part of David Véliz's PhD thesis, which aims at studying the respective role of both natural selection and larval dispersal in the genetic structure in marine invertebrates using *Semibalanus balanoides* as a model. D.V. is currently Instructor Professor at Universidad de Chile in Santiago, Chile. Pierre Duchesne's interests are in the development of new mathematical and computer tools for understanding genetic patterns, both at the levels of population and individual. Louis Bernatchez and Edwin Bourget supervised David Véliz's PhD thesis. L.B.'s interests relate to the understanding of patterns and processes of molecular and organismal evolution, and their relevance to conservation. E.B.'s interests are the study of the effects of environmental stress at different geographical scales in the intertidal species.

Appendix 1

Cohort 2000. Sample size (n), number of alleles per locus (A/L), gene diversity, observed heterozygosity (H_O: proportion of heterozygous individuals per sample), and F_{IS} according to Weir & Cockerham (1984) at different sites and microhabitats. *P* is the probability that F_{IS} are null. Significance of the exact test of HWE for microhabitat was tested using Fisher's method. LS, low sheltered; LE, low exposed; HS, high sheltered; HE, high exposed

	Burnt Church		Cap Lumière			
Location Sample	1 (LS)	2 (HS)	3 (LS)	4 (LE)	5 (HS)	6 (HE)
June						
n	29	29	52	57	52	52
A/L	9.4	8.8	9	9.6	9.6	10.2
H_E	0.71	0.72	0.69	0.67	0.71	0.73
H_O	0.55	0.61	0.61	0.62	0.62	0.59
F_{IS}	0.23	0.16	0.13	0.08	0.13	0.19
P	0.00	0.00	0.00	0.02	0.00	0.00
October						
n	25	23	61	76	49	35
A/L	6.4	7.2	9.6	11.2	9	8.2
H_E	0.70	0.69	0.71	0.71	0.73	0.72
H_O	0.54	0.64	0.65	0.67	0.64	0.64
F_{IS}	0.24	0.07	0.09	0.05	0.12	0.11
P	0.00	0.10	0.01	0.05	0.00	0.01

Appendix 2

Cohort 2001. Sample size (n), number of alleles per locus (A/L), gene diversity, observed heterozygosity (H_O: proportion of heterozygous individuals per sample), and F_{IS} according to Weir & Cockerham (1984) at different sites and microhabitats. *P* is the probability that F_{IS} are null. Significance of the exact test of HWE for microhabitat was tested using Fisher's method. LS, low sheltered; LE, low exposed; HS, high sheltered; HE, high exposed

	Le Goulet		Shippagan				Pointe Sapin				Cap Lumière			
Location	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sample	(LS)	(HS)	(LS)	(LE)	(HS)	(HE)	(LS)	(LE)	(HS)	(HE)	(LS)	(LE)	(HS)	(HE)
June														
n	31	24	22	20	31	31	31	22	27	32	24	32	30	31
A/L	8.8	7.6	7.4	7.2	9.8	8.6	7.6	6.8	5.4	8.2	7.6	8	7.4	8
H_E	0.74	0.73	0.75	0.71	0.74	0.72	0.72	0.68	0.68	0.71	0.72	0.69	0.72	0.71
H_O	0.58	0.60	0.70	0.66	0.67	0.58	0.53	0.60	0.51	0.53	0.60	0.65	0.52	0.66
F_{IS}	0.22	0.19	0.07	0.07	0.10	0.19	0.26	0.12	0.26	0.25	0.18	0.06	0.29	0.20
P	0.00	0.00	0.12	0.12	0.01	0.00	0.00	0.02	0.00	0.00	0.00	0.07	0.00	0.00

	1	2	3	4	5	6	7	8	9	10	11
Sample	(LS)	(HS)	(LS)	(LE)	(HS)	(LS)	(HS)	(LS)	(LE)	(HS)	(HE)
October											
n	32	25	30	28	30	32	29	29	31	26	30
A/L	8.8	7.6	7.8	8.6	6.8	8.8	8.8	7	7	6.4	8.2
H_E	0.74	0.73	0.69	0.72	0.70	0.74	0.73	0.68	0.70	0.70	0.73
H_O	0.59	0.53	0.65	0.60	0.50	0.60	0.64	0.53	0.51	0.64	0.53
F_{IS}	0.20	0.28	0.06	0.18	0.29	0.19	0.13	0.23	0.28	0.09	0.27
P	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00

Appendix 3

Local and global P *value calculation over grouped* r_{xy} *statistics*

To test the possibility that the grouping structure is statistically linked to relatedness as measured by the r_{xy} index, suppose that we have S groups of genotyped individuals: G1, G2, G3, … GS and the sizes of the groups are n1, n2, n3, … nS respectively and the total number of individuals is N. In other words, we seek to obtain a *P* value based on permutations of the N genotypes.

Here is a detailed description for one iteration of the permutation procedure:

1 Within each group and over all groups, compute the sum S_L of all r_{xy}(g1, g2) where g1 and g2 belong to the same group
2 Put together all genotypes from G1 … GS in a single list L of size N
3 Permute L randomly to obtain a new list P
4 Divide P into S groups of sizes n1 … nS identical to the sizes of the original groups
5 Compute the sum S_P within each group and over groups obtained from P as in 1)
6 If $S_P \geq S_L$ for some group or over all groups then add 1 to the corresponding counter variable C

To obtain a local or global *P* value the following three steps are performed:

1 C is set to 0
2 K iterations are run
3 *P* value = C/K