Molecular Ecology (2007) **16**, 1799–1810 doi: 10.1111/j.1365-294X.2007.03276.x

Finding the relevant scale: clonality and genetic structure in a marine invertebrate (*Crambe crambe*, Porifera)

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Abstract

Important changes in genetic relatedness may occur at extremely small scales in benthic invertebrates, providing key information about structuring processes in populations of these organisms. We performed a small-scale study of the population structure of the sponge *Crambe crambe*, in which 177 individuals from the same rocky wall (interindividual distances from 0 to 7 m) were genotyped using six microsatellite markers. 101 sponges had unique genotypes and the remaining 76 individuals formed 24 groups of sponges sharing genotypes (clones). Mean intraclone distances were found to be *c*. 20 cm. Spatial autocorrelation analyses showed a drastic decrease in genetic relatedness over the first 100 cm of distance. If the contribution of clonality to this pattern was eliminated, the trend was attenuated, but remained a marked one and was still significant within the first distance classes (30–40 cm). Estimated mean dispersal distances per generation were *c*. 35 cm, and neighbourhood sizes were estimated at *c*. 33 sponges. Genetic similarities with sponges of the same locality, or from other Mediterranean localities, were within the same range as those found in sponges 2–7 m apart. It is concluded that asexual reproduction plays an important role in structuring populations in this species. However, over and above the effects of clonality, a strong fine-scale genetic structure was present at distances in the range of tens of centimetres, probably as a result of short dispersal of larvae. This fine-scale genetic structure may be common in invertebrates with lecitotrophic larvae.

Keywords: clonal structure, kinship coefficient, microsatellites, neighbourhood size, spatial autocorrelation, sponge

Received 26 July 2006; revision received 22 November 2006; accepted 8 January 2007

Introduction

The contribution of asexual reproduction and restricted dispersal to the genetic structure of populations is an important question in evolutionary biology. In marine systems, where there is an apparent lack of barriers to gene flow, many studies have addressed the potential relationship between population connectivity and the dispersal capabilities of larvae and asexual propagules (see reviews in Grosberg & Cunningham 2001; Hellberg *et al*. 2002; Palumbi 2004). Most of the studies so far focus on the consideration of species as a set of subdivided populations occupying discrete areas in space, and measures of genetic differentiation between sites have been computed to obtain estimates of gene flow. The most likely situation, however, is that populations are not discrete, but continuous along whole stretches of coast. Treating populations as continuous allows the use of intrapopulation features such as autocorrelation data or neighbourhood sizes as descriptors of genetic structure and processes (Sokal & Oden 1978; Miller 1998; Hämmerli & Reusch 2003; Wagner *et al*. 2005). Spatial structuring can influence, and be influenced by, many important aspects of the biology of the species, particularly reproductive parameters (Sokal & Wartenberg 1983; Epperson & Li 1996) and can also have implications in conservation studies (Ruggiero *et al*. 2002;

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Marquardt & Epperson 2004). It seems reasonable, then, that investigations on the genetic structure of a species should firstly aim to determine the physical scale at which genetic structure is built.

Among the few groups of marine invertebrates in which intrapopulation genetic structure has been studied, cnidarians have received the most attention, often in the context of evaluating the role of clonal vs. sexual reproduction in the establishment of populations (e.g. Billingham & Ayre 1996; McFadden & Aydin 1996; McFadden 1997; Miller 1998; Adjeroud & Tsuchiya 1999). Information is also available on ascidians (Yund & O'Neil 2000) and sponges (see below). The latter is an ideal group for assessing spatial genetic structure at small scales, given that they are hermaphrodite, their larvae are short-lived (Maldonado & Bergquist 2002) and they have asexual reproduction capabilities (Wulff 1991). Some works have addressed the small-scale structure of sponges, mostly for clonality assessment, using histocompatibility tests (Neigel & Avise 1983; Neigel & Schmahl 1984), or allozyme electrophoresis (Davis *et al*. 1996; Whalan *et al*. 2005; Zilberberg *et al*. 2006), with contrasting conclusions as to the importance of asexual reproduction in the species studied.

We present here the first microsatellite-based spatial autocorrelation study of the small-scale genetic structure of a marine invertebrate. Our model organism was the Atlanto-Mediterranean encrusting sponge *Crambe crambe*. We selected this species because it is a major component of shallow sublittoral communities in the western Mediterranean (Uriz *et al*. 1992; references therein), it is well-known in terms of its biological characteristics (Turon *et al*. 1996, 1998; Becerro *et al*. 1997; Uriz *et al*. 1998), and its population differentiation has been studied with molecular tools (Duran *et al*. 2004a, b, c). Therefore, information from small-scale studies can be contrasted with predictions from life-history features and with among-population divergence data. Specifically, in a monitoring of a population of *Crambe crambe* over two years, Turon *et al*. (1998) found a moderate contribution of asexual reproduction (in this encrusting form, asexual reproduction is the result of fission of sponges) in the dynamics of the species: 14 fission events were observed in a monitoring of 75 sponges over two years.

Crambe crambe is a brooding species that releases fully developed larvae. Uriz *et al*. (1998) studied the field and laboratory behaviour of larvae of *Crambe crambe* and concluded that, compared to other sponge larvae, they are active swimmers that can move away from parental colonies. In another study, Mariani *et al*. (2006) showed that the abundance of larvae of *Crambe crambe* over sublittoral surfaces is not correlated to the small-scale (over several meters) distribution of adults. These results indicate that genetic structure due to restricted larval dispersal is not expectable at a scale of a few metres.

The objective of this work was to obtain data on spatial genetic structure at small scales in *Crambe crambe* to determine the contribution of asexual reproduction to the make-up of the populations, and to test the hypothesis that there should be no signature of restricted dispersal over scales of a few metres. Furthermore, we wanted to ascertain how within-population genetic variation compared to the between-population differentiation found in previous studies.

Materials and methods

Sampling

The study was conducted in the northwestern Mediterranean, at the locality of Tossa de Mar (NE of Spain, 41°43.2'N 2°56.4'E). The coast at this point consists of a rocky shore with vertical walls between 0 and 20 m deep. We selected a north-facing vertical wall running down to a sandy bottom 14 m below. This wall was inhabited by an algal assemblage representative of the zone. Details on the structure of the sublittoral communities in the locality can be found in Turon (1990).

Iron nails were hammered into the wall, delimiting the corners of a 3×3 m square extending from 4 to 7 m in depth, and another horizontal band 0.5 m high and 3 m wide located just below, at 10 m depth (Fig. 1a). This second zone was included in order to have a wider range of physical distances and to have more sponge pairs separated by distances in the range of 3–7 m. All sponges in these two areas (totalling 10.5 m^2) were counted for density estimation. For genotyping, as the total number of sponges was high (441 individuals), we selected a subset of the area (5.5 m^2) consisting of a cross design (Fig. 1a) of two orthogonal 3×1 m bands with the addition of the deeper 0.5×1 m band. In this way we covered the full range of distances available (from 0 to *c*. 7 m). To reproduce the exact size and position of every sponge in this area, we framed with nails the limits of the cross, and fixed to them tight vertical ropes which were used as guides for sliding horizontal ropes. Acetate sheets 20×30 cm in area were fixed to these horizontal ropes using pegs. Outlines of all the sponges present were drawn on the acetate sheets under water. The sliding ropes were moved downwards, new acetate sheets were placed on them, and outlines of another set of sponges were obtained. The process was repeated until we had a complete representation of the whole area on sheets. At the same time that a sponge was drawn, a small sample was collected and placed under water in numbered vials. 205 sponges were collected in this 5.5 m^2 area. Samples were fixed in absolute ethanol and stored at –20 °C.

The acetate sheets were digitized and mounted to reconstruct the sponge distribution on the studied surface. Unlike grid-based methods, which are constrained by the

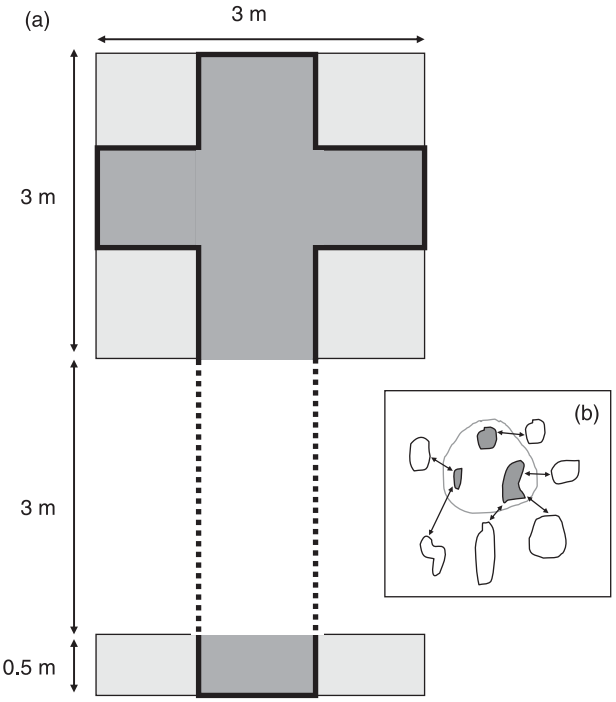

Fig. 1 (a) Sketch of the wall studied, with indication of the surfaces considered for density estimates (light grey), and for genotyping sponges (dark grey). (b) Illustration of the method used for recalculating distances between clones and other sponges. The three shaded sponges were assigned to a clone, and the minimal distance between any of them and the other sponges is recorded to reconstruct the distance matrix.

distance between grid points, we could calculate the exact distance separating individuals as we had a continuous representation of the wall. The global digital image was analysed with the IMAT software developed by the Scientific and Technical Services of the University of Barcelona. The software automatically computed the minimal distances between all pairs of sponges. As this species is known to reproduce asexually by fission (Turon *et al*. 1998), the shortest distances are the relevant ones for ascertaining the probability of clonal reproduction. The shortest distance between sponges also provides a conservative estimate of the reach of dispersal events of gametes and larvae.

Ramet and genet-level analysis

We will use the common terms of ramet and genet to refer to each physically separated sponge and to the group formed by sponges deriving asexually from a single original zygote, respectively (Jackson & Coates 1986). In order to eliminate the clonal structure from the spatial autocorrelation analysis (see below), we computed another set of distances in which sponges (ramets) sharing the same multilocus genotypes were treated as a single clonal sponge (genet). The distances were then recomputed so that the distance between a given genet and any other sponge was the shortest distance between this sponge and any of the ramets of the genet (Fig. 1b). This allowed us to eliminate the inflating effect of clonality on spatial autocorrelation measures while maintaining intact all the spatial information. For convenience, we will designate these distances as the genet-level dataset.

Computing the shortest distances to any member of the genet seems preferable to computing distances to a middle point of the genet because the latter alters the distance structure present in the original data (Reusch *et al*. 1999). There is no ecologic foundation in using a middle point (which is usually empty), while the shortest distance has a clear ecologic meaning: it is the minimal distance that a dispersal event should cover. We concede, however, that our estimates could be somewhat conservative for this reason. In order to evaluate the variance in the estimates resulting from the selection of different ways of measuring distances, a second genet-level dataset was computed using the geographic centre of the genets to calculate distances to other sponges. This was done automatically by the image analysis program, and the second dataset was submitted to the same autocorrelation analyses.

Genotyping

We used six microsatellite loci developed by Duran *et al*. (2002). These markers were shown to be unlinked and to have an appropriate level of variability (Duran *et al*. 2002). We used the same codes as in that study for designating the loci: *A* (CR1), *E* (CR43), *H* (CR59), *I* (CR83), *J* (CR91) and *K* (CR54). A certain number of sponges failed to amplify some of the loci even after at least three trials with different PCR conditions. We attributed these failures to null alleles. The percentage of non-amplifications varied among loci (1.1% in locus *A*, 6.1% in locus *E*, 6.5% in locus *H*, 0% in locus *I*, 2.3% in loci *J* and *K*). Although these percentages were low, in order to reduce errors in clone assignment and bias in genetic differentiation measures, individuals that did not amplify in all the loci were excluded from the analyses, thereby bringing the final number of sponges analysed down to 177, while noting that there is evidence for the existence of a certain amount of null alleles that can confound our estimates (see Discussion).

Genomic DNA was extracted using the REALPURE extraction kit (Durviz, Valencia, Spain). PCRs were performed in a final volume of 25 µL, using the primers and the protocol described in Duran *et al*. (2002). The annealing temperatures used are listed in Table 1. Allele sizes were estimated with an automated sequencer (ABI-Prism 3700, Applied Biosystems) relative to an internal size standard (EcoGen 70–500, EcoGen, Barcelona, Spain). Allele sizes were visualized and determined with GENESCAN™ version 3.7 and GENOTYPER™ version 3.7 software (Applied Biosystems).

Locus	T (°C)	# alleles	Sponge level (n = 177)			Genotype level (n = 125)		
			H_O	H_E	F_{IS}	H_O	H_E	F_{IS}
A	40	17	0.734	0.865	0.151*	0.712	0.872	0.184*
E	40	15	0.198	0.489	0.596*	0.200	0.526	0.621*
H	56	4	0.395	0.405	0.024	0.416	0.439	0.052
I	55	5	0.565	0.564	−0.002	0.616	0.619	0.005
J	50	7	0.627	0.787	0.203*	0.624	0.787	0.207*
K	56	9	0.689	0.725	0.049	0.656	0.713	0.080
Overall		9.5	0.536	0.640	0.163*	0.537	0.659	0.185*

Table 1 Characteristics of the the six loci studied: optimal annealing temperature and summary of genetic variation (H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient). Asterisks indicate significant values (at nominal P-value = 0.05, adjusted by sequential Bonferroni correction)

All alleles (with one exception) differed by at least two nucleotides. To avoid genotyping errors, all data were scored by the same person and were systematically double-checked to identify and eliminate errors that had occurred during data entry and scoring of alleles by hand. Samples with doubtful profiles, e.g. with poorly defined peaks or with peaks that were not observed in other profiles, were run a second time. Concordance of the allele sizes found with those from previous analyses in this species, run in the same machine (Duran *et al*. 2004b), reassured us that the incidence of genotyping errors, if any, is minimal in our dataset.

Clonality and spatial autocorrelation analyses

Observed and expected heterozygosities (Nei 1978), as well as inbreeding coefficients (following Weir & Cockerham 1984) and their significance level, were obtained using the GENEPOP computer program (Raymond & Rousset 1995), web version, and the FSTAT program version 2.9.3 (Goudet 2001).

For the study of the likelihood that any repeated multilocus genotype (MLG) could arise from sexual reproduction instead of representing true clones, we generated samples of the same size as the sample studied (177 in our case) by randomly selecting alleles using the observed frequencies, but maintaining the observed heterozygosity at each locus through an iterative algorithm. In this way, samples are obtained of exactly the same size, allele frequency and heterozygosity as the observed ones. This effectively mimics the effect of the factor(s) that can result in the observed heterozygosity level (null alleles and inbreeding, among others). After generating a large (10 000) number of samples, we recorded the proportion of samples that have any number of groups of identical MLG of any size. These proportions can then be used to test the likelihood of finding the observed patterns of identical MLG by chance if the population is reproducing only sexually, subject to the factor(s) that alter the expected heterozygosity level. We performed the simulation analyses using a modified version of the program presented in Duran *et al*. (2004b), written in Turbo Pascal by XT and available from the authors.

Spatial autocorrelation analyses were performed on the full (ramet-level) dataset and the genet-level dataset. We chose to use kinship coefficients (also called coancestry coefficients), which can be thought of as the probability that two alleles at an autosomal locus, one drawn randomly from each individual, are identical by descent (Ritland 1996; Van de Casteele *et al*. 2001). The use of kinship coefficients is preferable to other commonly used indices, such as Moran's I or Geary's c (Sokal & Oden 1978), as they have a sounder foundation in genetic theory and provide a natural means to summarize data over loci (Loiselle *et al*. 1995; Hardy & Vekemans 1999; Vekemans & Hardy 2004). We used the index described in Loiselle *et al*. (1995) which is not dependent on Hardy–Weinberg (HW) equilibrium conditions (Hardy & Vekemans 1999; Fenster *et al*. 2003) and is shown to perform best among commonly used indices in determining spatial genetic structure (Vekemans & Hardy 2004). The reference population for the estimation of allele frequencies was the whole sample. We also used the 'a' index proposed by Rousset (2000) as a measure of genetic distance between individuals. This index has important advantages: it does not depend on a particular sample and does not require a reference population (Vekemans & Hardy 2004). Moreover, it is analogous to the $F_{ST}/(1 - F_{ST})$ ratio commonly used when regressing genetic divergence with distance (Rousset 2000), so it is appropriate for comparisons at larger scales (see below). On the other hand, it has the inconvenient of suffering higher variance than the kinship coefficients (Vekemans & Hardy 2004). We will use the notations F_{ij} and a_{ij} for the kinship coefficient and the Rousset index, respectively, between sponges i and j. We ran the analyses using the SPAGEDI 1.2 program (Hardy & Vekemans 2002), which automatically combines data over alleles and loci and performs sampling bias corrections.

We chose the distance categories of the spatial autocorrelation analyses as a compromise between the spatial resolution and the number of pairs in each category. We used tighter classes at short distances and wider ones at longer distances: every 10 cm from 0 to 1 m, every 20 cm from 1 to 2 m, then one metre (from 2 to 3 m), then 2 m

(from 3 to 5 m) and then from 5 m to the maximal distance recorded, 6.8 m. These distances designate the end-points of the distance classes, and the plots were drawn using the mid-point of each. We had 15 576 pairs of sponges in the full dataset and 7750 pairs in the gene-level dataset. The minimum number of pairs in any distance class of both datasets was 115 (with a mean of 678). For the genet-level analysis using the mid-point of the genets for computation of distances there were less pairs in the shortest range of distances, so we collapsed the first three distance classes into two: 0–15 cm and 15–30 cm, in order to have more than 100 pairs in every distance class.

Furthermore, we estimated the variance of the indices at each distance class through jackknifing. The 95% confidence intervals of the expected index values if there is no particular association at a given distance interval were calculated through randomizations (1000) of the dataset permuting the location of the individuals using the built-in permutation options of SPAGEDI. We also performed a regression of all points on distance or log distance, and tested the significance of the slope through randomization. We used these slopes to estimate gene-dispersal parameters (see below).

The same indices used for interindividual comparison can be used at larger spatial scales by defining groups in the SPAGEDI program and restricting the analyses to between-group pairings of sponges. In this way we were able to ascertain the degree of differentiation within the wall studied relative to larger spatial scales. The work by Duran *et al*. (2004b) using the same loci in the same species provided material for comparison. In that work several populations were sampled (with sample sizes of *c*. 30 sponges) in different localities of the Atlanto-Mediterranean arch.

Inferences on gene-dispersal distances and neighbourhood sizes

Information of gene flow in continuous populations is summarized in the parameter of neighbourhood size (*Nb*) which, in a two-dimensional space, is calculated as $Nb = 4\pi D\sigma^2$, where D is the effective (breeding) population density and σ^2 is the variance in dispersal between parents and offspring along a single axis passing through the population (axial variance) (Rousset 1997; Fenster *et al*. 2003; Vekemans & Hardy 2004). In a two-dimensional space, the second moment of the euclidean dispersal distance is twice the axial variance ($2\sigma^2$) (Crawford 1984; Rousset 1997). As the distribution has an expected zero mean (i.e. no net displacement of the population over time), then $2\sigma^2$ equals the mean squared euclidean dispersal distance in two dimensions. *Nb* is a good indicator of the balance between local genetic drift and gene dispersal within continuous populations (Vekemans & Hardy 2004) and is a convenient indirect measure of gene flow (Fenster *et al*. 2003; Alberto *et al*. 2005). The neighbourhood area is defined as the circular area that contains *Nb* individuals.

Nb can be estimated from the slope of the regression line between genetic coefficients and distance. In particular, for kinship coefficients $Nb \approx -(1 - F_{IS})/blog$ (Fenster *et al*. 2003; Heuertz *et al*. 2003), where *blog* is the slope of the regression with the natural logarithm of physical distances, and F_{IS} is the inbreeding coefficient. For Rousset's index, $Nb \approx 1/blog$ (Rousset 2000). However, these relationships hold only within a range of distances that depends on σ and the mutation rate of the marker used (Rousset 1997). For microsatellites, a convenient range is from σ to 20σ (Heuertz *et al*. 2003). In the absence of any independent information about dispersal distances, this leaves two variables to estimate (σ and *Nb*), and only one regression. We used an iterative procedure to simultaneously estimating σ and *Nb* (Vekemans & Hardy 2004). It starts by estimating *Nb* from the global regression slope, then calculating σ from this *Nb*, then restricting the regression to distances between σ and 20σ, and estimating again *Nb* and *s*. The procedure is repeated until σ converges (convergence is not assured, though, e.g. Heuertz *et al*. 2003). This procedure is implemented in SPAGEDI for the kinship coefficient, but not for Rousset's coefficient. For the latter we performed the steps of the iteration manually, taking advantage of the restricted range regression option in SPAGEDI.

The effective density measures should take into account the variability in reproductive success, which is rarely known in natural populations. We will use here the census density in the wall studied as the best proxy for effective density. In this species, most individuals of a wide range of sizes are reproductive during the spring–summer season (Uriz *et al*. 1995).

Another commonly used measure is the intercept of the spatial autocorrelation function (or their confidence intervals, Hämmerli & Reusch 2003) with the *x*-axis. This measure has been generally called patch size (Sokal 1979) and is interpreted as a measure of the spatial scale of autocorrelation (Epperson & Li 1997). Although the use of neighbourhood sizes is preferable to the use of patch sizes (Vekemans & Hardy 2004), one useful related measure is the so-called clonal subrange (Alberto *et al*. 2005), which is the point where the spatial autocorrelation function considering all individuals and the one excluding clonality intersect. The clonal subrange can be used as a descriptor of clonal structure and is equivalent to the distance where the probability of clonal identity approaches zero (Alberto *et al*. 2005).

Results

The six microsatellite loci studied had between 4 (locus H) and 17 (locus A) alleles in the sample studied (average 9.5). The main measures of genetic variation are presented for

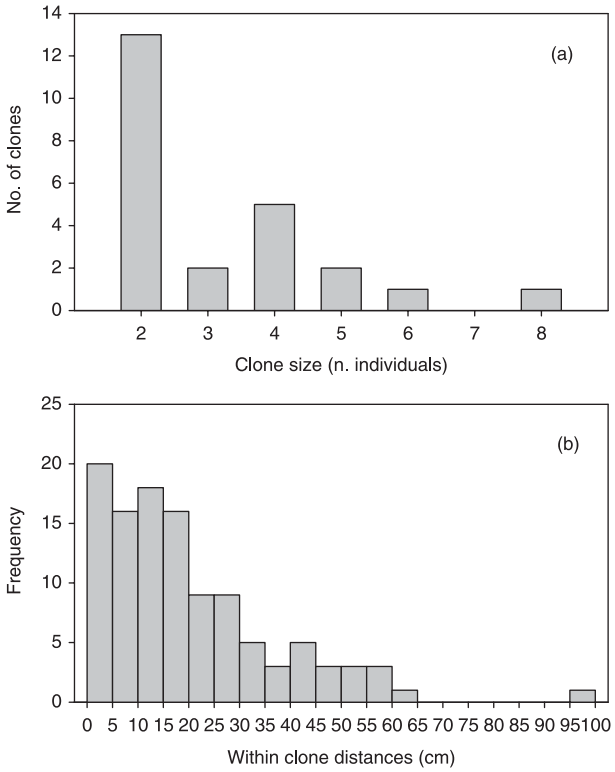

Fig. 2 (a) Frequency distribution of the sizes (in number of sponges) of the 24 groups of sponges sharing multilocus genotypes (genets). (b) Frequency distribution of the distances between sponges sharing genotype.

the whole sample of individual sponges and at the genet level (with only one representative of each MLG) in Table 1. The observed heterozygosities were in most cases smaller than expected, and the F_{IS} values were significant in three out of six loci at both the individual and genet levels. Global F_{IS} values were 0.163 and 0.185, respectively. These values are significant and indicated departure from HW conditions in our samples.

We found clear evidence for clonal structure in our samples. Seventy-six out of 177 sponges had genotypes shared by at least two individuals. There were 24 different shared MLG. Most frequently only two sponges comprised the shared MLG, but up to eight sponges with the same genotype have been found (Fig. 2a). When we ran the simulation of 10 000 samples of 177 sponges with the allele frequencies and heterozygosity levels observed in our sample, less than one group of identical multilocus genotypes appeared per sample (0.35 ± 0.006 mean ± SE). Furthermore, we can know exactly the distribution of sizes of the groups of identical MLG that appeared: we found one group of two identical MLG in 23.4% of the samples, two groups of two identical MLG in 4.7% of the samples, three groups of two identical MLG in 0.7% of the samples, one group of three identical MLG in 0.4% of the samples. Other combinations appeared in 0.06% or less of the samples. Using these percentages as probability estimators, we can conclude that, given the allele frequencies and heterozygosity level observed, one can reasonably expect to find a group of two identical MLG by chance in a sexually reproducing population (23.4% probability), while finding more than that is unlikely (less than 5% probability of any combination). In short, sexual reproduction cannot explain the pattern of 24 groups of identical MLG found. We therefore considered these as genets resulting from asexual reproduction, assuming just a modest possibility of error (likely one out of 24 'clones'). It is informative in this sense that the frequency of homozygotes in the repeated genotypes is not higher than in the unique genotypes (paired-sample *t*-test locus by locus, $P = 0.148$), which indicates that the excess of homozygosys in the samples did not influence the appearance of identical MLG. Further evidence comes from the spatial distribution of the ramets: the distance between sponges sharing the same genotype was 19.97 ± 1.6 cm (mean ± SE), which was significantly lower (Mann–Whitney test, $P < 0.001$) than distances between sponges that do not share genotypes (200 ± 2.39 cm). The frequency distribution of the distances (Fig. 2b) reveals that most ramets of a genet were separated by less than 65 cm with only one pair separated by 95 cm.

Spatial autocorrelation analyses at both ramet and genet level show strong spatial structuring (Fig. 3) over the first distance classes, indicated by a steep decrease of the similarity index (or an increase of the distance index) in the first metre. For the ramet-level analysis, the intersection of the autocorrelation line with the 95% confidence interval envelope occurred at the 60 cm distance class (F_{ij}) and at the 50 cm class (a_{ij}) (note that we refer to distance classes by their end-points). These distances are clearly coincident with the range of distances separating clonemates. To eliminate the influence of clonality, we performed the genet-level analysis and found an appreciable decrease of the similarity index (and increase of the distance index): for the first distance class, the F_{ij} index fell from 0.247 to 0.158, while a_{ij} increased from –0.140 to 0. Nevertheless, a significant structure was still present in the first distance classes, and the intersection of the genet-level autocorrelation line with the corresponding 95% interval line occurred at distance classes of 40 cm (F_{ij}) and 30 cm (a_{ij}). Once the autocorrelation values entered the 95% confidence interval, they remained within it at larger distances, except for a few points (Fig. 3). When the genet-level analyses was performed using the mid-point of genets instead of the shortest distances, the resulting autocorrelation lines were almost identical, with a slight right-displacement in the first distance classes (Fig. 3). The intersection with the corresponding 95% interval line (not shown in Figure) was at 50 cm for both indices in the dataset using mid-points of genets.

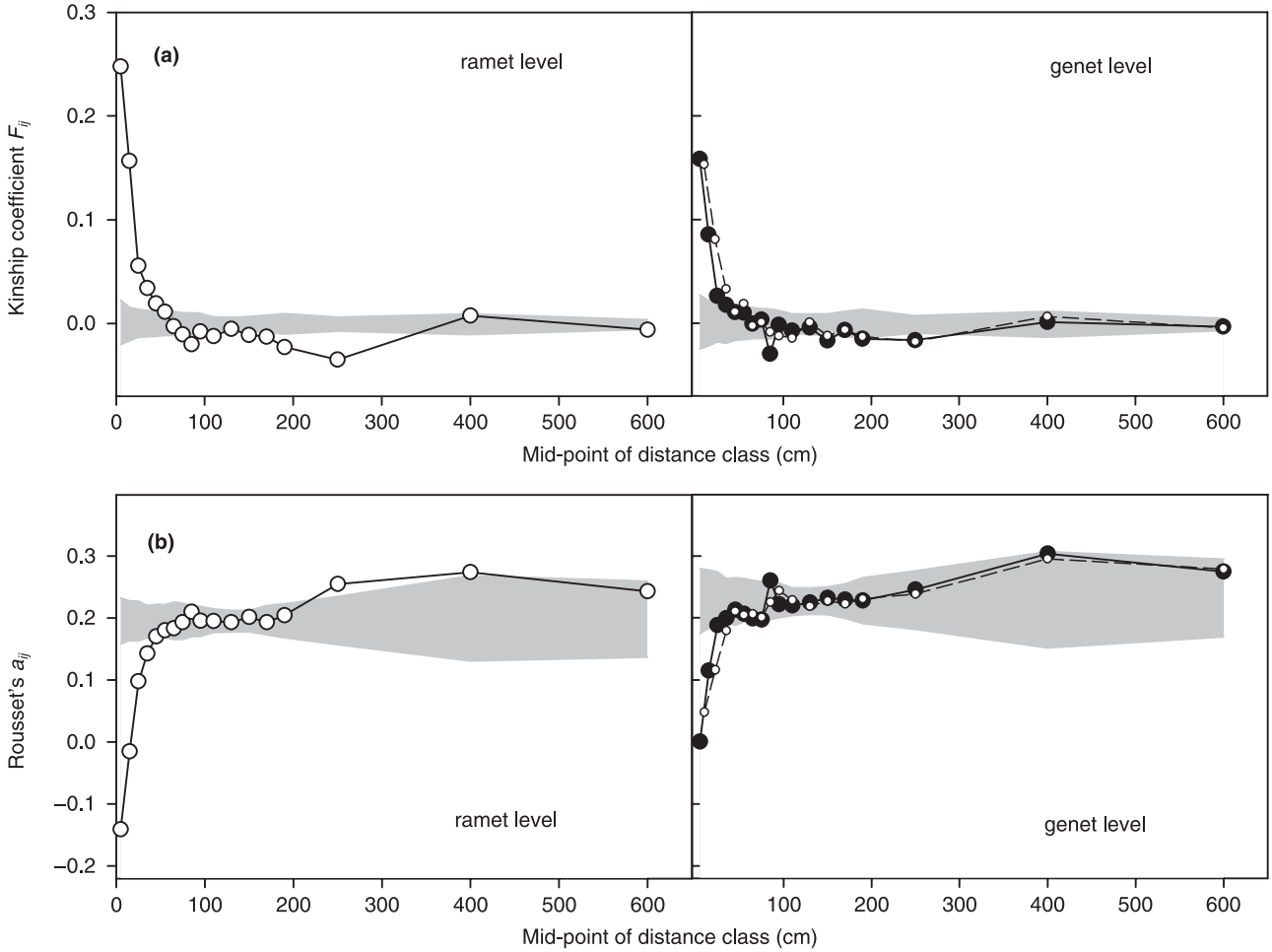

Fig. 3 Plots of the spatial autocorrelation curves of the kinship coefficient (a) and Rousset's distance (b) with physical distance. Both the ramet and genet-level analyses are represented. The shaded area delimits the 95% interval of no association obtained through randomization. In the genet-level analyses, the dotted line represents the autocorrelation line using distances to the physical centre of the genets rather than shortest distances.

The clonal subrange, or the point at which the autocorrelation lines of the ramet and genet-level analyses intersect for the first time, was at the distance classes of 70 cm (F_{ij}) and 80 cm (a_{ij}) (irrespective of the use of shortest distances of mid-points of genets). At these distances the probability of clonal identity, estimated as the proportion of pairs within the corresponding distance class that include sponges with the same genotype (Alberto *et al*. 2005) fell below 0.002. This probability was maximal at the shortest distance class (10 cm, 0.18), fell below 0.05 at the 30 cm class, and was 0 from the 100 cm class onwards.

We attempted to estimate the dispersal parameter σ, as well as the neighbourhood size (*Nb*), from the slopes of the autocorrelation coefficients with the natural logarithm of distance using the iterative procedure described above. The regression slopes for both indices were significantly different from 0 (randomization procedure implemented in SPAGEDI). In our calculations we used the census density, estimated as 42 sponges m^{-2} in the total area where sponges had been counted (10.5 m^2). These analyses were performed only at the genet level to avoid the confounding effect of clonal structure. For the kinship coefficient F_{ij} the values did not converge because the slope turned to positive values (indicating infinite neighbourhoods) after a few iterations. For Rousset's distance we obtained convergence and were able to estimate a neighbourhood size (32.88 sponges) and a σ parameter (24.84 cm). In order to examine the influence of using shortest distances to a clone on these parameters we did the same calculations using distances to the centre of the clones, and obtained slightly smaller estimates (σ = 23.79 cm, *Nb* = 29.86 sponges) due to a bit steeper slope with the logarithm of distance.

To compare the degree of differentiation within the wall studied relative to larger spatial scales, we used data from

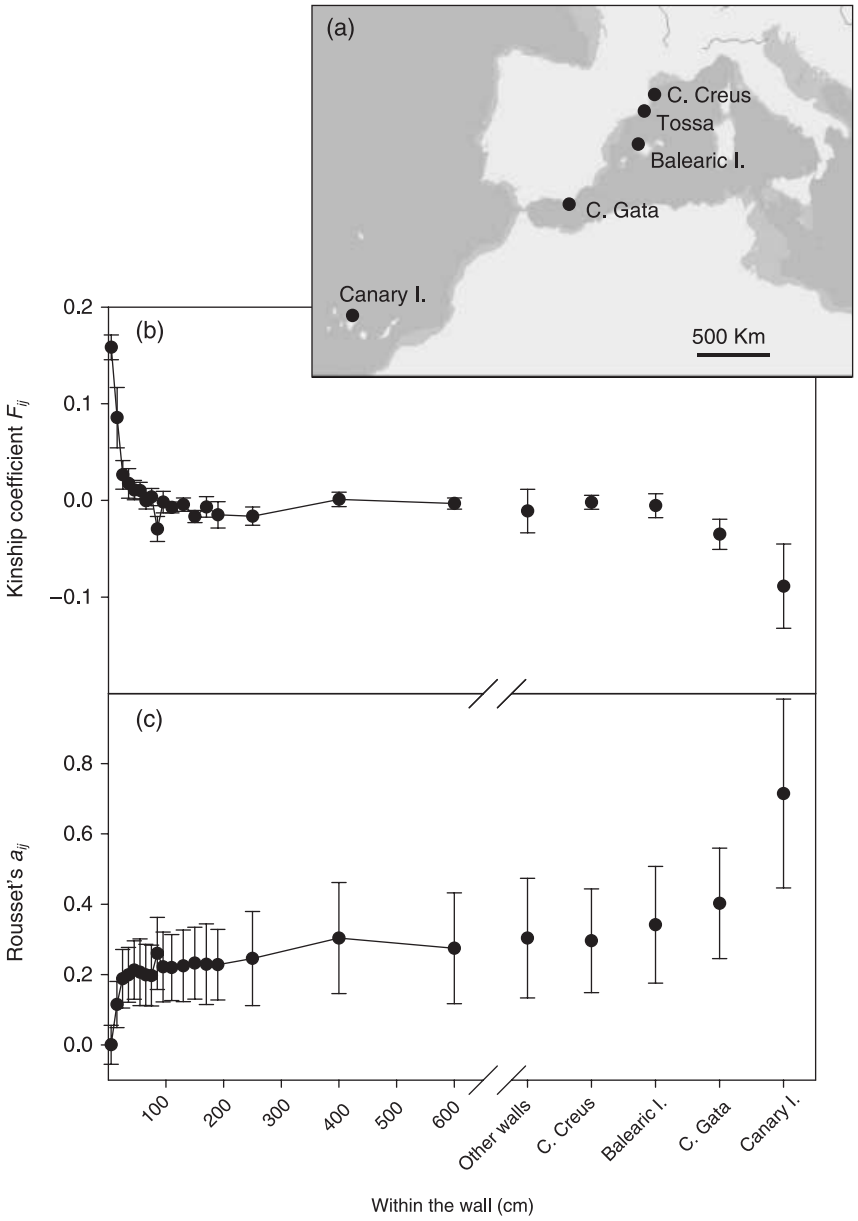

Fig. 4 (a) Location of the studied locality (Tossa) and the other populations in the Atlanto-mediterranean area used for comparison. Comparison of the kinship coefficient (b) and Rousset's distance (c) within the wall studied and with sponges from other walls of the same locality and from the other localities. Error bars are SE estimates obtained through jackknifing over loci.

Duran *et al*. (2004b) from different populations of *C. crambe* in the Atlanto-Mediterranean arch (Fig. 4). To begin with, sponges from the same locality of Tossa de Mar had been genotyped. They belong to different walls from the one studied here, thus providing a spatial scale of tens of metres. We also selected samples from other localities in order to have a wide range of distances: Cap de Creus (separated *c*. 70 Km), Balearic Islands (*c*. 300 Km), Cabo de Gata (*c*. 700 Km) and Canary Islands (*c*. 2300 Km, all shortest distances by sea). The results obtained by applying the F_{ij} and a_{ij} indices to pairings formed between the sponges in the wall and sponges of the locality being compared are shown in Fig. 4. Results obtained within our wall (genet-level analysis) are also represented for comparison, as well as standard errors of the estimates obtained through jackknifing over loci. The reference population for calculation of allele frequencies in the F_{ij} index was the whole sample combining all localities. It can be seen that, for distances up to hundreds of Km, the F_{ij} indices remained close to the values obtained within the wall at distances larger than a couple of metres. In other words, sponges separated by several metres are as unrelated between them (i.e. F_{ij} close to 0) as sponges separated by many kilometres, only the comparison with the Cabo de Gata sample and, especially, with the Canary Islands sample showed a clear decrease in the kinship coefficient, with negative values (indicating allelic differentiation between localities). The distance index a_{ij} showed the same pattern,

but its increment was more gradual with distance. It seems therefore that the steep increase in genetic differentiation found at scales of 1 m or less is unparalleled by the degree of differentiation between populations unless one moves to distances in the order of thousands of kilometres.

Discussion

In this article we have studied the small-scale genetic structure of a sessile invertebrate in an effort to show the relative importance of phenomena occurring at distances from centimetres to a few metres in explaining the genetic make-up of this species. Our results clearly indicated that asexual multiplication plays an important role in the structuring of the populations of the sponge *Crambe crambe*. Seventy-six out of 177 sponges (43%) have genotypes shared by at least two individuals. The close spacing between sponges sharing genotypes (mean distance of 19.97 cm) supports the theory that fission events are the main asexual reproduction method (Turon *et al*. 1998). Clonal subranges were of the order of 70–80 cm, indicating that clonality as an effect on the structure of populations at distances below 1 m.

We found a strong spatial genetic structure when analysing all sponges with autocorrelation techniques, with significant values of the indices used at distances under 50–60 cm. However, it is crucial to distinguish between the effect of clonal structure and limited gene flow in the spatial autocorrelation values (Reusch *et al*. 1999). In the literature, this has been done in different ways, generally leading to reduced data sets that eliminate all but one representative of each clone. Only among clones can genetic diversity related to drift and recombination be assessed (Reusch *et al*. 2000). In our case, we included the repeated MLG only once in the analyses, but we kept the full geographic information by recalculating distances between genets and the remaining sponges. When we ran the spatial autocorrelation analyses with the restricted dataset (genet-level analysis), the strength of the autocorrelations decreased. Nevertheless, significant values were still found at the smallest distance classes, up to 30–50 cm depending on the index used and the method for calculating the distance. The results remained essentially the same using the shortest distance to any ramet of the genet or the distance to the mid-point of the genet. Several studies on marine organisms report that spatial autocorrelation values were no longer significant when changing from the ramet to the genet level of analysis (McFadden & Aydin 1996; Reusch *et al*. 1999; Van der Strate *et al*. 2000), indicating that most of the spatial structure was contributed by the proximity between clonemates. In our case, however, a significant amount of structure persisted, suggesting that isolation by distance acts at small scales. The limited dispersal found should be the result of short dispersal of both sperm and larvae.

The existence of a certain amount of null alleles in our sample (see Material and Methods) can affect our results (Selkoe & Toonen 2006). However, although the absolute values of the coefficients can be biased, the overall shape of the autocorrelation curve will not be affected, as there is no reason to suspect that the null alleles are not uniformly distributed over the area studied. The significance levels obtained are not affected either, as the randomization procedure incorporates the potential effects of these null alleles in the allele frequency distribution. Therefore, although kinship values may be somewhat inflated to the extent that null alleles contribute to the excess of homozygotes, the overall conclusions of these analyses remain unaffected.

A crucial point is the confidence level in the assignment of individuals sharing multilocus genotypes to clones. Several methods have been reported to estimate the likelihood of shared MLG arising as a consequence of asexual reproduction vs. the alternative that they are the result of sexual reproduction (e.g. Parks & Werth 1993; Reusch *et al*. 2000; Waits *et al*. 2001; Stenberg *et al*. 2003). A common problem with these different approaches is the underlying assumption of random mating. As deviations from HW equilibrium are commonplace, the different estimates may suffer from severe biases. In particular, the usual deficit of heterozygotes in populations with some degree of inbreeding makes the appearance of repeated MLG more likely than the different methods estimate. Our method of simulating populations of the same heterozygosity level as the one studied does not have this problem. For our population, this method indicates that sexual reproduction makes a negligible contribution to the observed pattern of repeated MLG, which should be the result of asexual reproduction.

It may be argued that, if the population consists of patches of interbreeding individuals, the allele frequencies that should be taken into account when examining the probability of shared MLG are the local allele frequencies of the patch, rather than the population allele frequencies. We tested this effect for the biggest genet found (comprising eight ramets). We recomputed the probabilities of finding shared MLG using the allele frequencies of all the sponges located at a distance of less than 1 m from any of the eight ramets (61 sponges in total). The simulation showed a low probability (4.2%) of finding any shared MLG (and in all cases these were of size 2). Hence, even considering the local allele frequencies of our biggest genet, the appearance of a pair of sponges with the same genotype, let alone eight sponges, is unlikely to be the result of sexual reproduction within the sponge genetic patch.

The kinship coefficient between mates is the expected inbreeding coefficient of their outcrossed descendence (Fenster *et al*. 2003). In this sense, it is informative to compare

the overall inbreeding coefficient with the kinship coefficient among the most closely related sponges (i.e. those at the smallest distance class). In our case, the overall inbreeding coefficient at the genet level was 0.185 ± 0.07 (SE obtained by jackknifing over loci), while the kinship coefficient at distance class 1 was 0.158 ± 0.01. The 95% confidence intervals did not overlap, so the two values were significantly different. In other words, biparental inbreeding among sponges, even among those most similar in our population, does not suffice to explain the overall inbreeding level. As clonality has been excluded in this dataset, this is strong indication of the existence of a certain degree of selfing among the sponges (Fenster *et al*. 2003). However, this result should be taken with caution as we do not know the relative influence of null alleles in our kinship and inbreeding coefficients.

The axial dispersal parameter σ has been estimated to be *c*. 24–25 cm according to the method used. As σ^2 is half the mean squared physical distances between parents and offspring (Crawford 1984; Fenster *et al*. 2003), these values of σ correspond to a gene displacement of 34–35 cm per generation. This distance encompasses dispersal due to both sperm and larvae. Of course, the distribution of dispersal distances is probably highly leptokurtic, so a mean value may not give an adequate idea of occasional long-distance events. However, it is clear that this overall restricted dispersal is enough to build structure at rather small scales: the calculations of neighbourhood sizes gave values of *c*. 30–33 sponges. Given the observed density of 42 sponges m^{-2}, this means that the genetic neighbourhood area is somewhat less than 1 m^2. It should also be considered that we have no estimates of effective density, and to the extent that census density exceeds effective density, our results overestimate *Nb*. Direct measures of dispersal distances in this species are lacking, but reports in other groups of benthic invertebrates indicate extremely localized dispersal of sperm, over scales of just a few tens of centimetres (Pennington 1985; Grosberg 1991; Yund 1995). Sponge larvae are lecitotrophic and short-lived, which restrict their dispersal capabilities (Maldonado & Bergquist 2002). To our knowledge there are no direct studies of distances that larvae of sponges can travel, but for other groups of benthic invertebrates with lecitotrophic larvae, direct and indirect (rare allele markers) methods have revealed dispersal distances of the order of centimetres or a few metres (reviewed in Shanks *et al*. 2003). Our dispersal estimates agree with the idea of philopatric dispersal, with at least some larvae settling very close to parent sponges. Mean asexual and sexual dispersal distances in this species are of the same order of magnitude, and both contribute to the strong structure found at distances of less than 1 m.

The small scale differentiation found here within a single wall is of the same order as that found over very large scales, up to hundreds of kilometres. This indicates the importance of factors acting at restricted scales (e.g. local inbreeding and drift, asexual reproduction etc.) compared with those acting at long distances (e.g. occasional long-distance gene flow, mutation-drift equilibrium etc.). Similar divergence levels between populations separated by hundreds of metres and hundreds of kilometres were also found in the soft coral *Alcyonium rudyi* by McFadden (1997). The genetic differentiation found when comparing with samples from the Canary Islands agrees with the phylogeographic studies that inferred a colonization event from the Mediterranean with a corresponding bottleneck effect (Duran *et al*. 2004a, 2004b).

Although caution must be observed as we have examined only one population of the sponge, in the case of *Crambe crambe* asexual reproduction and restricted dispersal, possibly coupled with some degree of selfing, seem to contribute to the strong genetic structure found at the intrapopulation level. The microevolutionary implications are important, as the existence of spatial genetic structure means possibilities for local adaptations and can contribute to the small-scale variability observed in many biological parameters of this species (e.g. Uriz *et al*. 1995; Becerro *et al*. 1997).

The finding of important clonal structure and restricted dispersal of larvae contradicts the predictions made from biological studies. The key to explain this discrepancy, in our view, lies in the low growth rates and high longevity of *Crambe crambe*, which imply that the processes that built the genetic structure of the wall studied may well have been acting over decades. Although a monitoring of two years (Turon *et al*. 1998) revealed only a moderate frequency of fission events, these events accumulated over the years giving rise to the clonal structure detected. Likewise, the studies of Uriz *et al*. (1998) and Mariani *et al*. (2006) showed that larvae of *Crambe crambe* can effectively disperse over distances of several metres, especially so in rough sea conditions. However, for a build-up of spatial genetic structure to occur, it suffices that a fraction of the larvae released settle in the vicinity of the parental sponge. Our results call for caution when drawing conclusions from temporally restricted biological or demographic studies, which represent just a snapshot of long-term processes. As the genetic structure preserves the past history of the population, thereby integrating information over successive generations, genetic tools are useful to assess patterns and processes that may go undetected in other kinds of study. Assessing the spatial scale or scales at which genetic variation occurs is a crucial first step necessary for adequate planning of studies on biology, demography, gene flow or conservation of marine invertebrates. Failure to identify the relevant scales can result in waste of time and money and, worse still, in reaching the wrong conclusions.

Acknowledgements

We wish to thank the staff of the Scientific and Technical Services of the University of Barcelona for their help with image analysis and genotyping procedures. We also thank Natàlia Sant, Cruz Palacín and Isabel Tarjuelo for their help with the field work. This research was funded by project CTM2004-05625 of the Spanish Ministry of Science.

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