

Editorial Manager(tm) for Marine Biology
Manuscript Draft

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Title: Microscale genetic differentiation in a sessile invertebrate with cloned larvae: investigating the role of polyembryony

Article Type: Original Study

Keywords: polyembryony; cloned larvae; *Crisia denticulata*; microsatellites; spatial autocorrelation; AMOVA.

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Abstract: Microscale genetic differentiation of sessile organisms can arise from restricted dispersal of sexual propagules, leading to isolation by distance, or from localised cloning. Cyclostome bryozoans offer a possible combination of both: the localised transfer of spermatozoa between mates with limited dispersal of the resulting larvae, in association with the splitting of each sexually produced embryo into many clonal copies (polyembryony). We spatially sampled 157 colonies of *Crisia denticulata* from subtidal rock overhangs from one shore in Devon, England at a geographic scale of c. 0.05 to 130m plus a further 21 colonies from Pembrokeshire, Wales as an outgroup. Analysis of molecular variance (AMOVA) revealed that the majority (67%) of genetic variation was distributed among individuals within single rock overhangs, with only 16% of variation among different overhangs within each shore and 17% of variation between the ingroup and outgroup shores. Despite local genetic variation, pairwise genetic similarity analysed by spatial autocorrelation was greatest at the smallest inter-individual distance we tested (5cm) and remained significant and positive across within-overhang comparisons (<4m). Spatial autocorrelation and AMOVA analyses both indicated that patches of *C. denticulata* located on different rock overhangs tended to be genetically distinct, with the switch from positive to negative autocorrelation, which is often considered to be the distance within which individuals reproduce with their close relatives or the radius of a patch, occurring at

the 4-8m distance class. Rerunning analyses with twenty data sets that only included one individual of each multilocus genotype (n=97) or the single data set that contained just the unique genotypes (n=67) revealed that the presence of repeat genotypes had an impact on genetic structuring (PhiPT values were reduced when shared genotypes were removed from the dataset) but that it was not great and only statistically evident at distances between individuals of 1-2m. Comparisons to a further twenty randomisations of the data set that were performed irrespective of genotype (n=97) suggested that this conclusion is not an artefact of reduced sample size. A resampling procedure using kinship coefficients, implemented by the software package GENCLONE gave broadly similar results but the greater statistical power allowed small but significant impacts of repeat genotypes on genetic structure to be also detected at 0.125-0.5 and 4-16m. Although we predict that a proportion of the repeat multilocus genotypes are shared by chance, such within-overhang distances may represent a common distance of cloned larval dispersal. These results suggests that closely situated potential mates include a significant proportion of the available genetic diversity within a population, making it unlikely that, as previously hypothesised, the potential disadvantage of producing clonal broods through polyembryony is offset by genetic uniformity within the mating neighbourhood. We also report an error in the published primer note of Craig et al. (2001, *Molecular Ecology Notes*, 1: 281-282): loci Cd5 and Cd6 appear to be the same microsatellite.

Reply to Marine Biology reviewers comments
Ms. No. MABI-D-06-00478
Authors. Pemberton et al.

Dear Prof. Wahl and Marine Biology editorial team,

Please find attached our revised manuscript following your email of 22 March 2007. I am sorry it has taken me a few weeks to get the manuscript back to you. There were a greater number of modifications to be made than I first thought, plus my wife gave birth to our first child a few weeks back and I have suddenly been rather busy!

As requested we have addressed each point raised by the reviewers. To make this as clear as possible we have copied and pasted the entirety of your email of 22nd March in uppercase below and have inserted our responses in lowercase. References to the line numbers in the original submission have been supplemented with [old] to differentiate them from line numbers in the revised manuscript, which are prefixed with [new] (e.g. Lines [new] 1-4).

Yours sincerely,

Andrew Pemberton.

REF.: MS. NO. MABI-D-06-00478
MICROSCALE GENETIC DIFFERENTIATION IN A SESSILE INVERTEBRATE WITH
CLONED LARVAE: INVESTIGATING THE ROLE OF POLYEMBRYONY
MARINE BIOLOGY

DEAR DR PEMBERTON,

REVIEWERS HAVE NOW COMMENTED ON YOUR PAPER. YOU WILL SEE THAT THEY ARE ADVISING THAT YOU REVISE YOUR MANUSCRIPT. IF YOU ARE PREPARED TO UNDERTAKE THE WORK REQUIRED, I WOULD BE PLEASED TO RECONSIDER MY DECISION.

FOR YOUR GUIDANCE, REVIEWERS' COMMENTS ARE APPENDED BELOW. PLEASE PAY PARTICULAR ATTENTION TO THE COMMENTS OF REVIEWER #3: HE WILL PROBABLY CONSIDER YOUR PAPER A SECOND TIME.

IF YOU DECIDE TO REVISE THE WORK, PLEASE SUBMIT A LIST OF CHANGES OR A REBUTTAL AGAINST EACH POINT WHICH IS BEING RAISED WHEN YOU SUBMIT THE REVISED MANUSCRIPT.

PLEASE LET US HAVE YOUR REVISION AS SOON AS POSSIBLE.

TO SUBMIT A REVISION, GO TO [HTTP://MABI.EDMGR.COM/](http://MABI.EDMGR.COM/) AND LOG IN AS AN AUTHOR. YOU WILL SEE A MENU ITEM CALL SUBMISSION NEEDING REVISION. YOU WILL FIND YOUR SUBMISSION RECORD THERE.

YOURS SINCERELY

MARTIN WAHL, PH.D.
REGIONAL EDITOR
MARINE BIOLOGY

REVIEWERS' COMMENTS:

REVIEWER #1: THIS STUDY PRESENTS SMALL-SCALE (150 M MAXIMUM) GENETIC VARIATION IN THE CYCLOSTOME BRYOZOAN CRISIA DENTICULATA (EGG-

BROODING, FREE-SPAWNING) USING FIVE MICROSATELLITE MARKERS TO TEST RYLAND'S (1996) ASSUMPTION REGARDING THE ROLE OF POLYEMBRYONY. POLYEMBRYONY INVOLVES SPLITTING SEXUALLY PRODUCED EMBRYOS INTO SEVERAL CLONAL COPIES WITHIN ZOOIDS. ANIMAL POLYEMBRYONY APPEARS TO BE PARADOXICAL BECAUSE AN UNPROVEN GENOTYPE IS CLONED AT THE EXPENSE OF GENETIC DIVERSITY (CRAIG ET AL. 1996). ON THE OTHER HAND, SOME REPORTS OF SPERM LIMITATION IN MARINE ORGANISMS HAVE SUGGESTED A POSSIBLE RESOLUTION OF THIS PARADOX, IN THAT IT MAY BE BETTER TO PROVIDE FEWER EGGS AND GREATLY REPLICATE ANY THAT ARE FERTILIZED (E.G., RYLAND 1996). THE PRESENT STUDY TESTED THE BASIS OF RYLAND'S HYPOTHESIS THAT THE RELATIVE LOSS OF OFFSPRING GENOTYPIC DIVERSITY FROM POLYEMBRYONY WOULD NOT DIFFER GREATLY FROM THAT OF SEXUAL REPRODUCTION BECAUSE AVAILABLE SPERMATOZOA ORIGINATE FROM SIMILAR BACKGROUNDS BY INBREEDING (RYLAND 1996). ALTHOUGH SPATIAL AUTOCORRELATION ANALYSIS SHOWED THAT POPULATIONS OF THIS SPECIES LOCATED IN DIFFERENT ROCK OVERHANGS WERE LIKELY GENETICALLY DISTINCT, AN ANALYSIS OF MOLECULAR VARIANCE (AMOVA) REVEALED THAT THE MAJORITY OF GENETIC VARIATION WAS DISTRIBUTED AMONG INDIVIDUALS WITHIN SINGLE OVERHANGS. THESE RESULTS CONTRADICT RYLAND'S ASSUMPTION ABOUT THE GENETIC HOMOGENEITY IN LOCAL POPULATIONS OF THIS SPECIES.
Summary of the study. No response needed.

MY FIRST IMPRESSION IS THAT RYLAND'S ASSUMPTION MAY BE AN EXTREME CASE WHEREIN CLONES THAT ARE PRODUCED THROUGH POLYEMBRYONY ARE UNLIKELY TO DIFFER FROM SEXUAL BROODS (IN HIS WORDS, "POTENTIAL MATES WILL DIFFER LITTLE"). THIS ASSUMES THAT THE POPULATION MUST BE EXTREMELY INBRED WITH A SMALL POPULATION SIZE, AND/OR STRICTLY ISOLATED AND SUBJECTED TO THE FOUNDER EFFECT. THE PRESENT STUDY DEMONSTRATED THAT LOCAL POPULATIONS OF THIS SPECIES DID NOT MEET THESE ASSUMPTIONS; THEREFORE, IT IS IMPORTANT TO SHOW EMPIRICALLY THAT GENETIC POPULATION ANALYSIS DATA DID NOT SUPPORT RYLAND'S ASSUMPTION.
This appears to be a comment only, so does not need a change to the manuscript.

I THINK ONE OF THE MOST IMPORTANT PROBLEMS IN THIS POPULATION ANALYSIS IS THE SAMPLE SIZE AND SAMPLING STRATEGY. THE AUTHORS COLLECTED SAMPLES RANGING FROM 9 TO 50 INDIVIDUALS FOR EACH OVERHANG (MEAN: 16.2), WHICH WAS TOO SMALL. THE SAMPLE SIZES FOR POPULATION GENETIC ANALYSES HAVE TYPICALLY BEEN 30-50 IN OTHER PUBLISHED STUDIES THE AUTHORS MIGHT HAVE BEEN ABLE TO COLLECT MORE SAMPLES IN SOME OVERHANGS EXCEPT L AND M, WHICH HAD NOT ENOUGH INDIVIDUALS (ELECTRONIC SUPPLEMENTARY MATERIAL S2C)...

Our sample sizes would be on the small side for traditional population genetic analyses where allelic frequencies are being used to give a population average against which to compare to other populations. We have not tried to hide the numbers used (e.g. Table 1) and had already included a warning paragraph on this topic (lines [old] 167-174). Such a power problem is of course taken into account by the statistics and, as outlined in Table 3, we seem to have sufficient power to detect significant genetic differentiation in most pairwise overhang comparisons. It is of course true that some of the non-significant comparisons in Table 3 could be the result of a lack of power and Referee 1 is right to highlight this point. To make this clearer to the reader we have added a sentence to the table legend for Table 3 ("Some caution is required in interpreting these pairwise overhang comparisons as sample size varied as described in Table 1.") and to the new summary table (see Reviewer #2 section below). We have also modified the main text slightly. However the most relevant rebuttal to this point is that the backbone of this study, and the method around which the sampling strategy was designed, is not the population (i.e. overhang) based statistics but the spatial autocorrelation. Spatial autocorrelation is an individual pairwise technique where the statistical power comes from the number of pairwise comparisons in each spatial class (the values above the x-axis in Figure 1). The clustering of these individuals into human-defined groups is irrelevant for the

power of the test. The results, as shown in Figure 1, clearly demonstrate that we have sufficient power to detect significant spatial genetic structure.

Reviewer #1 paragraph continues... ON THE OTHER HAND, THE AUTHORS CREATED A VIRTUAL SAMPLE DATA SET ASSUMING THAT THE PRESENT DATA SET WAS FOR THE MOTHER POPULATION, WHICH SEEMED TO BE APPROPRIATE FOR SOME OVERHANGS. HOWEVER, THERE MAY HAVE BEEN SOME BIAS IN HIGH-DENSITY OVERHANGS BECAUSE THE COLLECTED GRID SAMPLES APPEARED TO BE NONRANDOM IN SPECIFIC OVERHANGS, ESPECIALLY G AND O. SOME OF THE MOST AGGREGATED INDIVIDUALS WERE NOT COLLECTED IN THOSE OVERHANGS (ELECTRONIC SUPPLEMENTARY MATERIAL S2C). THE AUTHORS MAY NEED TO ANALYZE HIGHLY AGGREGATED INDIVIDUALS, WHICH WOULD BE USEFUL FOR CLARIFYING WHETHER GENETIC HOMOGENEITY IS ALSO EVIDENT IN THOSE INDIVIDUALS.

Now that we have the results of the current study, which describe spatial genetic structuring at remarkably small geographic distances, it is clearly in our mind to go back to the overhangs and carry out sampling at an even smaller geographic scale. Obviously when we planned the study we did not know the results and executed the sampling on what seemed sensible from the literature in terms of what to expect for genetic structuring and estimates of distances over which sperm could be transferred and to give a good spread of pairwise distance classes to generate meaningful spatial autocorrelation correlograms. We agree with Referee 1 that centimetre scale mapping and genotyping in the most dense stands of *Crisia* would be interesting but we hope you agree that it would be a separate piece of follow-up work rather than something essential for this publication.

RYLAND (1996) ARGUED THAT OWING TO THE LIMITED DISPERSAL OF BOTH SPERM AND BROODED LARVAE, POTENTIAL MATES WILL DIFFER LITTLE, AND SEXUAL REPRODUCTION MAY PRODUCE LARVAE WITH GENOTYPES NO LESS FIT THAN THEIR PARENTS WITHIN THE IMMEDIATE VICINITY. BECAUSE IT IS DIFFICULT TO ESTIMATE THE DIFFERENTIATION OF FITNESS BETWEEN GENOTYPES, I THINK IT IS REASONABLE THAT THE AUTHORS FOCUSED ON THE DEGREE OF GENETIC VARIATION AMONG POTENTIAL MATES AT SMALL SCALES. THEIR STUDY SUCCESSFULLY SHOWED THAT GENETIC VARIATION WAS MAINLY DISTRIBUTED AMONG INDIVIDUALS WITHIN POPULATIONS (OVERHANGS), ALTHOUGH EACH POPULATION DIFFERED GENETICALLY. THE SMALL SAMPLE SIZE IS AGAIN PROBLEMATIC FOR THE HW TEST AND GENETIC POPULATION STRUCTURE; HOWEVER, THE RESULTS OF THIS STUDY ON THE MICROSACLE GENETIC VARIATION (LACK OF EXTREME GENETIC HOMOGENEITY) IN *C. DENTICULATA* ARE IMPORTANT AND PROVIDE BASIC INFORMATION FOR UNDERSTANDING OF ROLE OF POLYEMBRYONY. GIVEN THAT FEW MICROSACLE GENETIC STUDIES OF MARINE POLYEMBRYONIC SPECIES HAVE BEEN PUBLISHED, I THINK MARINE BIOLOGY IS A SUITABLE JOURNAL FOR THIS STUDY.

No response needed.

SEE BELOW FOR SPECIFIC COMMENTS.

ABSTRACT

OVERALL, THE ABSTRACT SHOULD BE SHORTENED. I WOULD SUGGEST DELETING LINES 2-10 AND 25-28, WHICH ARE UNNECESSARY IN THE ABSTRACT. HOWEVER, THE AUTHORS SHOULD POINT OUT DETAILED DATA AND RESULTS OF SOME ANALYSIS. FOR EXAMPLE, THE SAMPLE SIZE, NUMBER OF UNIQUE GENOTYPES, AND PERCENT DISTRIBUTION FOR EACH ?RT, ?PR, AND ?PT ARE NECESSARY TO UNDERSTAND THIS STUDY. IN ADDITION, THE AUTHORS SHOULD ALSO SHOW THE DATA SETS AND RESULTS OF THE SPATIAL AUTOCORRELATION ANALYSIS. THERE WERE FOUR TYPES OF DATA SET: ONLY UNIQUE GENOTYPES, ONE OF EACH GENOTYPE, 97 RANDOM INDIVIDUALS, AND ALL COLLECTED 178 (OR 157) INDIVIDUALS. THE RESULTS SHOULD INDICATE THAT MORE VARIATION WAS FOUND WITHIN POPULATIONS AND LESS AMONG POPULATIONS IN THE 'ONE OF EACH

GENOTYPE' DATA SET COMPARED TO THE 'FULL WEMBURY' DATA SET, WHICH DOES NOT APPEAR TO BE DUE TO THE REDUCED SAMPLE SIZE.

We have done our best to do all the above. Shortening the abstract whilst including all the requested information is not so easy of course! Lines [old] 2-10 have been contracted. Lines [old] 25-28 have been removed (somewhat reluctantly - see Reviewer #2's comments). In summary all that was asked for is now included (i.e. sample size, number of unique genotypes, results of the AMOVA and descriptions of the four data sets).

INTRODUCTION

THE INTRODUCTION ALSO SEEMED TO BE RATHER LENGTHY AND SHOULD BE SHORTENED...

We have moved the vast majority of the [old] second paragraph, which provided background biology on bryozoans, into the Materials and Methods section. This shortens the Introduction by about a quarter.

Paragraph continues... I SUGGEST THAT THE THIRD PARAGRAPH BE INCORPORATED INTO THE LAST ONE.

As suggested we have incorporated the [old] third and [old] final paragraphs. However as the [old] third paragraph forms an important bridge between the introduction of polyembryony itself (first paragraph) and the introduction of which aspects of the biology would be relevant to a study of small scale genetic differentiation in a polyembryonic species (remaining paragraphs) we have moved the [old] final paragraph into the [old] third paragraph rather than vice versa. To make this link clearer we have added a few sentences to the end of the [new] second paragraph (= [old] third paragraph).

MATERIAL AND METHODS

L. 155: WHY DID YOU BEGIN ON A NEW LINE? I THINK THIS SHOULD BE INCLUDED IN THE PREVIOUS SENTENCE.

Done.

L. 160: SIXTY PCR CYCLES (AUTHORS FOLLOWED HUGHES ET AL. 2005) SEEMS EXCESSIVE; GENERALLY ONLY 25-40 CYCLES ARE USED. MORE CYCLES WOULD RESULT IN DECREASED ACTIVITY OF DNA POLYMERASE AND INCREASED PCR ERRORS. I AM CONCERNED THAT THE AUTHORS FOUND SO MANY PEAKS.

We agree with Reviewer #1 that this number of cycles is probably excessive and that future workers could almost certainly reduce the number. The main response is however that despite having a lot of cycles the protocol worked, was repeatable, and gave biologically believable results (in the Hughes et al 2005 study we had known mother-offspring comparisons so could check inheritance patterns).

As to why we ended up with such a protocol the brief history is that we had some problems adapting the protocols from the Mol Ecol Notes primer note (which used a Pharmacia ALFexpress sequencer) to work on our Licor sequencer and suspected that steric hindrance of the different fluorescent primers was at least partly to blame. This led us to try a touchdown PCR protocol with only one sixth of the forward primer concentration being fluorescently labelled to try to kick start the amplification process with unlabelled primers so that the labelled primers could then amplify off amplicons where steric hindrance would be expected to be less of a problem. Having a greater number of cycles in a touchdown protocol is normal as one is unsure of the temperature at which annealing will begin and want to leave sufficient cycles at the lowest annealing temperature to get a good yield. We were also trying to get the protocol working on embryonic samples so were aware that template concentrations may have been low. What almost certainly happened is that we tried something like the current protocol with lots of cycles so we could exclude cycle number as a possible reason for PCR failure. The protocol worked, and worked well, and we never attempted to reduce the cycle number.

L. 196: I COULD NOT UNDERSTAND THIS CALCULATION: $217 \times 33^6 \times 7^8 = 1.9 \times 10^{13}$. THE AUTHORS SHOULD PROVIDE A MORE DETAILED EXPLANATION.

This calculation was meant to give the reader an idea of the vast number of possible

randomisations of the data set that would be possible. However, it was really not necessary and broke up the flow of the text somewhat. It has been removed.

RESULTS

L. 267: DOES '90 COLONIES' REFER TO THE NUMBER OF INDIVIDUALS OF REPEAT GENOTYPES? PLEASE PROVIDE A MORE THOROUGH EXPLANATION.

The sentence was not completely clear as it stood. Adding the word "shared" seems to sort it out.

L. 282-294: WHERE ARE THE DATA? THE AUTHORS SHOULD PROVIDE A TABLE OR FIGURE NUMBER, OR INDICATE 'DATA NOT SHOWN' IF THIS IS THE CASE.

All data are given in the text. We did not want to present the same data in both a table and the text and thought this was the clearest way to present the information as we could give the numbers and describe their meaning at the same time. It would of course be simple to repeat the data in a table such as the one below (again all data just copied from the text). Let me know if you would like this done.

Data set	Phi PT	% variation within / among overhangs
Full Wembury data set	0.1998	80 / 20
One of each genotype	Mean 0.1621, SD 0.0090	83-87 / 13-17
Random n=97	Mean 0.1972, SD 0.0210	76-83 / 17-24
Unique genotypes only	0.1428	86 / 14

DISCUSSION AND CONCLUSIONS

THE FIRST, SECOND, AND FOURTH PARAGRAPHS MENTION THE USEFULNESS OF SPATIAL AUTOCORRELATION STATISTICS FOR GENETIC STRUCTURE ANALYSIS. THE THIRD PARAGRAPH DISCUSSES THE RESULTS OF SPATIAL VARIATION (AMOVA), BUT NOT AUTOCORRELATION STATISTICS. THUS, THE AUTHORS FIRST MENTION SPATIAL AUTOCORRELATION RESULTS, AND THEN DISCUSS THE AMOVA. I WOULD SUGGEST REPLACING THE THIRD PARAGRAPH WITH THE FOURTH ONE. ADDITIONALLY, THE FIRST AND SECOND PARAGRAPHS ARE RELATED TO EACH OTHER, SO I SUGGEST THAT THESE TWO PARAGRAPHS BE MERGED INTO ONE. All done (i.e. the re-ordering of paragraphs [old] 3 and 4 that and amalgamation of paragraphs [old] 1 and 2).

TABLE 1: I SUGGEST THAT NG:N (PROPORTION OF UNIQUE GENOTYPES TO THE NUMBER OF INDIVIDUALS) VALUES SHOULD BE ADDED TO TABLE 1, WHICH WILL BE USEFUL FOR UNDERSTANDING GENOTYPIC DIVERSITY IN EACH OVERHANG. THE NG:N VALUES DIFFERED AMONG OVERHANGS, RANGING FROM 0.2 (OVERHANG J) TO 0.75 (OVERHANG G). THESE DATA INDICATE THAT THE DEGREE OF SUCCESSFUL POLYEMBRYONY DIFFERED ON A SMALL ENVIRONMENTAL SCALE.

Done.

TABLE 2: OVER 40% OF GENETIC VARIATION WAS DISTRIBUTED AMONG REGIONS IN LOCUS CD7. THIS LOCUS WAS THE MOST ALLELIC AMONG THE FIVE LOCI, BUT THE DATA ARE CONTRARY TO THE INTERPRETATION OF THIS STUDY. HOW DO YOU RECONCILE THIS?

As expected from this result locus Cd7 showed a very different allelic frequency distribution in the 21 Pembrokeshire samples (the outgroup region) than in the 157 Wembury samples (the ingroup region). Specifically allele 172 made up 83% of alleles in the 21 Pembrokeshire samples but only 16% of alleles in the Wembury samples. Similar differences were of course not present in the other loci. Thus there would appear to be macro-scale genetic differentiation between the two regions, which would not be surprising given what is known about the biology of this species and the micro-scale patterns reported in this study. However what the current study focuses on is micro- rather than macro- processes and it is genetic

differentiation between overhangs within the single shore of Wembury that we are interested in. If, instead of looking at the results from the “Full Wembury + Pembrokeshire data set” as shown in Table 2, we perform the same analysis on just the “Full Wembury data set” (i.e. we exclude the Pembrokeshire samples) we find that all five loci give very similar partitions of variation within and among overhangs as shown below:

Locus	PhiPT	% of variation apportioned within overhangs
Cd4	0.252	75
Cd5	0.235	76
Cd7	0.160	84
Cd8	0.089	91
Cd15	0.289	71

Thus data from all loci individually are in agreement with our interpretation of the study that within a shore most of the variation is found within overhangs. We had somewhat ignored this part of the data in the original version of the manuscript but now include it to clear up similar ‘worries’ from future readers. Table 2 and the text have been modified.

TABLE 3: READERS MAY MORE EASILY UNDERSTAND THE RELATIONSHIP BETWEEN GENETIC STRUCTURE AND GEOGRAPHIC FACTORS IF THE AUTHORS MERGE DATA FROM TABLE 3 AND S2B...

We could add the aerial photograph to the main text but then would we also need the other photograph of S2A? It seems somewhat of an overkill although we have no problem with this at all. For now we have left the order largely as it stood but have added another sentence to the Table 3 legend to remind the reader of the existence of S2B. We would of course be happy to change it if this is the editorial decision.

Paragraph continues...WHAT ARE D, E, AND F IN S2B?

Our apologies. D, E and F are other overhangs that we sampled for non-genetic purposes. They are included in a separate study that addresses how population density influences brood number (the brief answer is that it does not by the way!). We would like to keep these labels where they are if possible to allow us to cross-reference between studies. If this is not acceptable we can certainly delete them. We have added a sentence to the legend accompanying S2B to make this clear.

S1: DOES SELF-FERTILIZATION GENERALLY OCCUR IN THIS SPECIES? THIS INFORMATION SHOULD BE PROVIDED.

It seems most appropriate to add this information to the section where bryozoan biology is introduced. This has been done.

S3.1: READERS WILL MORE EASILY UNDERSTAND THE INFORMATION OF DOMINANT ALLELES AND ALLELE NUMBERS IN EACH OVERHANG IF THE AUTHORS PROVIDE A MORE DETAILED TABLE IN WHICH ROWS = LOCI AND ALLELE NUMBERS AND COLUMNS = OVERHANGS.

Done. We have inserted a table of allelic frequencies for each overhang as Table S3.2.

REVIEWER #2: THE PAPER BY PEMBERTON ET AL. REPORTS MICROSATELLITE DATA FROM CYCLOSTOME BRYOZOAN COLONIES ALONG A SCALE OF GEOGRAPHIC DISTANCE FROM 0.1 TO MORE THAN A 100 METERS. THE DATA SUGGEST SMALL-SCALE DISPERSAL OF SPERM AND/OR LARVAE OF CRISIA DENTICULATA ON THE ONE HAND AND THE MAINTENANCE OF SUBSTANTIAL GENETIC DIVERSITY WITHIN A SMALL SCALE „POPULATION“ (OVERHANG) ON THE OTHER. THE SUM OF DATA PROBABLY REJECTS RYLAND'S (1996) EXPLANATION OF POLYEMBRYONY, BUT I FIND THE LATTER DIFFICULT TO UNDERSTAND HERE. THE EXPERIMENTAL DESIGN, THE DATA AND THE ANALYSES LOOK SOLID AND THE OUTLOOK THAT WE ARE A

LONG WAY FROM BEING ABLE TO QUANTIFY THE PRECISE COSTS OF POLYEMBRYONY IS CERTAINLY ALSO SOLID TRUTH. THIS IS A VERY NICE PAPER THAT CAN ALMOST BE PUBLISHED AS IS.

MAYBE JUST A FEW THINGS FOR THE REVISIONS:

I WOULD FIND A TABLE LISTING THE DIFFERENT HYPOTHESES (IN BIOLOGICAL TERMS) TESTED WITH EACH STATISTICAL TEST VERY HELPFUL FOR THE GENERAL READER.

Done. This is quite a sizable table so has been added to the ESM as S8. If you prefer, it could easily go in the main text.

THE CITED GENETIC UNIFORMITY WITHIN A SINGLE BROOD CAN ONLY BE TRUE IF NOT MORE THAN 1 EGG IS FERTILIZED AND DEVELOPED PER GONAZOID AND BROOD. IS THIS THE CASE FOR ALL CYCLOSTOME BRYOZOANS? IF YES, PLEASE MENTION THIS FOR THE GENERAL READER.

The answer is not known as only this species has been analysed with molecular markers. A sentence has been added to the 'Bryozoan biology' section to make this clear.

REPEAT GENOTYPES DON' T HAVE TO BE CLONEMATES OR FULL- OR HALF-SIBLINGS.

Yes. The original sentence was imprecise. We have changed it.

ABTSR., 2ND. PAR., 1ST SENT.: PLEASE REPHRASE FOR SIMPLICITY. TO ME THIS IS A LITTLE-CONVINCING ARGUMENT ANYHOW.

The second paragraph, first sentence of the abstract stated "Colonies of *Crisia denticulata* inhabit subtidal rock overhangs." This does not seem controversial. We think Reviewer #2 meant the first sentence of the third paragraph. We have rephrased it as requested and concur that our data do not back up the idea.

SINCE THE AUTHORS DO NOT MEASURE ANY COSTS OF CLONAL PROPAGATION BY POLYEMBRYONY I FIND THE GENERAL DISCUSSION OF ITS RELATIVE ADVANTAGES AND DISADVANTAGES A LITTLE OVERDONE.

Removing the "Bryozoan biology section to the Materials and Methods" has streamlined the Introduction and we hope the Reviewer agrees that this section is now much easier to read.

IN SUM, VERY NICE PAPER.

REVIEWER #3: THIS IS A POTENTIALLY VERY INTERESTING PAPER, DESCRIBING THE POPULATION GENETIC CONSEQUENCES OF POLYEMBRYONY IN BRYOZOANS. THE WORK IS TECHNICALLY SOUND, ALTHOUGH SOME OF THE DATA ANALYSIS NEEDS TO BE IMPROVED, AND THE INTERPRETATION NEEDS TO BE PUT INTO A BROADER CONTEXT BEFORE THE PAPER BECOMES ACCEPTABLE IN MAR BIOL.

MY COMMENTS FOLLOW IN ORDER OF IMPORTANCE.

1 DATA ANALYSIS:

- HAVE THE AUTHORS EXCLUDED MULTIPLY SAMPLED INDIVIDUALS BEFORE CONDUCTING AMOVA? OTHERWISE RESULTS ARE BIASED BECAUSE SOME INDIVIDUALS ENTER TWICE (OR EVEN MORE OFTEN).

Yes. We performed AMOVA analyses on all four data sets. The results made up a whole paragraph (lines [old] 282-294) and remain unchanged. We had not included this data in the Abstract or Discussion sections however (which may be why the reviewer missed it). We have changed this now.

SECONDLY, THE LIKELIHOOD THAT TWO GENOTYPES ARE IDENTICAL BY CHANCE, AND NOT DUE TO SPLIT EMBRYOS, OBVIOUSLY AN ESSENTIAL PIECE OF INFORMATION FOR INTERPRETING THE RESULTS, CAN BE CALCULATED, SEE PARKS, J.C. & WERTH, C.R. (1993) A STUDY OF SPATIAL FEATURES OF CLONES IN A POPULATION OF BRACKEN FERN, PTERIDIUM AQUILINUM (DENNSTAEDTIACEAE).

AM. J. BOT., 80, 537-544.

A NICE SOFTWARE THAT DOES ALL THIS IS PROVIDED BY ARNAUD-HAOND, S. & BELKHIR, K. GENCLONE: A COMPUTER PROGRAM TO ANALYSE GENOTYPIC DATA, TEST FOR CLONALITY AND DESCRIBE SPATIAL CLONAL ORGANIZATION. MOL ECOL NOTES, IN PRESS

ONCE THIS ERROR-LIKELIHOOD IS ASSESSED, THE AUTHORS CAN ATTRIBUTE IDENTICAL GENOTYPES TO CLONALLY PRODUCED IDENTICAL EMBRYOS (E.G. LINE 391).

We thank the Reviewer #3 for this useful suggestion and the recommendation of this very interesting piece of software that we were not aware of. It has been implemented.

-FINALLY, A MORE MEANINGFUL STATISTIC TO COMPUTE SIMILARITY AMONG GENOTYPES WOULD BE A KINSHIP COEFFICIENT, SUCH AS THE ONE PROPOSED BY LOISELLE (SEE FOR AN EXAMPLE HÄMMERLI, A. & REUSCH, T.B.H. (2003) GENETIC NEIGHBOURHOOD OF CLONE STRUCTURE IN EELGRASS MEADOWS QUANTIFIED BY SPATIAL AUTOCORRELATION OF MICROSATELLITES. HEREDITY, 91, 448 - 455.)

We performed analyses using the kinship coefficient formulae of Loiselle (as suggested) and that of Ritland (1996, *Genetical Research* 67:175) on the 'Full Wembury data set' and found that the correlograms are very similar to the originals shown in Figure 1A. On further investigation of the GENCLONE software (see above) we found a very nice routine that automatically performs the kind of randomisations we carried out manually for our 'One of each genotype' data sets. We performed this analysis and two other autocorrelation procedures adapted to the existence of replicate genotypes within the sample (a weighted approach of Alberto et al 2005) and using central coordinates for each group of repeat genotypes (e.g. Hammerli & Reusch 2003). All tests are described in the manuscript with a new Figure 2 and [new] S5 of the ESM.

2 I DISAGREE THAT CLONAL STRUCTURE HAS ONLY A MINOR ROLE ON GENETIC STRUCTURE (E.G. IN ABSTRACT). COMPARE PANELS A AND D OF FIG. 1: THE POSITIVE SPATIAL AUTOCORRELATION IS ENTIRELY DRIVEN BY CLONAL STRUCTURE, POSSIBLY RESULTING FROM POLYEMBRYONY. THIS RESULT NEEDS TO BE STRENGTHENED

Reviewer 3 is correct to point out that Figures 1A and 1D are different, but we must disagree that this means that clonal structure had a major role on genetic structure. Comparing figures 1A (the full Wembury data set of 157 colonies that includes clones) to Figure 1D (the most severe contraction of the data set leaving only 67 colonies) is not the key comparison that one must make. The 'unique genotypes only' data set of Figure 1D, as the name suggests, only includes unique genotypes i.e. it excludes all genotypes that were found more than once - so if genotype x was found three times, then all three colonies are excluded from the analysis! It really is a harsh contraction, but is useful in that there is only one way of doing it - unlike the contraction of the data set for figure 1B. For the comparison that Reviewer 3 seeks, one must first compare Figures 1A with 1B (not 1D). Figure 1B, again as the name suggest, contains just 'one of each genotype'. So in the example above of genotype x which was found three times, then here we would leave just one of those genotypes in the data set (unlike in 1D where they were all excluded). The problem of course is which one of the three to leave in and this is why we ran twenty randomly generated data sets to get a feel for how the choice of which repeat genotype to include altered the analyses. As outlined above we have now included a further similar autocorrelation analysis of the resampling approach of GENCLONE. This permutation technique has considerable power and was able to detect significant differences between correlograms when repeat genotypes were included or excluded that were not apparent in the original analyses. These have been incorporated into the text so that we now conclude that in this study the strength of within-overhang genetic similarity was increased by a small but significant amount by the presence of repeat multilocus genotypes at the distance classes of 0.125-0.5, 1-2 and 4-16m. In summary, our conclusions are now more similar to what Reviewer 3 suggested although this is derived from a different test.

3 THE STUDY DESIGN IS NOT SUITED TO DETECT ANY COSTS OR BENEFITS OF POLYEMBRYONY. RATHER, IT EXAMINES ITS EXTENT AND THE POPULATION

GENETIC CONSEQUENCES IN TERMS OF SPATIAL GENETIC STRUCTURE. THE INTRO AND DISCUSSION NEED SOME REFORMULATION INTO THIS DIRECTION...

We are keen to keep the primary focus on polyembryony, but we have reformatted the text to take this view into account.

Paragraph continues... AN INTERESTING RELATED ANALYSIS WOULD BE TO ASSESS IF THERE IS EVIDENCE FOR LOCALIZED POCKETS OF INBREEDING WHERE POLYEMBRYONY IS HIGH, I.E. WHERE RATIOS OF OUTCROSSING DROP, RATHER THAN LOOK AT MEAN GENETIC DIVERSITY. MAYBE SUCH AN ANALYSIS IS POSSIBLE, TOO (SEE FOR A COMPARISON ANOTHER PLANT PAPER: REUSCH, T.B.H. (2001) FITNESS-CONSEQUENCES OF GEITONOGAMOUS SELFING IN A CLONAL MARINE ANGIOSPERM (ZOSTERA MARINA). J. EVOL. BIOL., 14, 129-138)

We thank the reviewer for this suggestion for future work.

4 THE PAPER WOULD BENEFIT FROM A BROADER FOCUS. FOR EXAMPLE, SESSILE PLANTS ALSO HAVE SIMILAR 'EVOLUTIONARY' PROBLEMS, FOR EXAMPLE THE PROBLEM OF OUTCROSSING WHEN CLONES GET TOO BIG (SEE HANDEL, S.N. (1985) THE INTRUSION OF CLONAL GROWTH PATTERNS ON PLANT BREEDING SYSTEMS. AMER. NAT., 125, 367-384).

We were keen to keep a tight focus on polyembryony in this paper and had avoided much of the broader literature where it did not seem directly relevant. However there is no harm in some broader focus and as requested we have incorporated other literature as requested.

METHODOLOGICALLY, WHAT THE AUTHOR HAVE DONE HAS MANY INTERESTING PARALLELS WHAT RESEARCHERS IN THE (MARINE) CLONAL PLANT WORLD HAVE ALREADY DONE: COMPARING AUTOCORRELOGRAMS WITH AND WITHOUT IDENTICAL GENOTYPES (=POTENTIAL CLONE MEMBERS, SEE ABOVE). THE AUTHORS MAY WISH TO CITE THE HAEMMERLI PAPER GIVEN ABOVE WHO DID THIS FOR ONE OF THE FIRST TIMES.

Done.

Title: Microscale genetic differentiation in a sessile invertebrate with cloned larvae:
investigating the role of polyembryony

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spatial autocorrelation, AMOVA.

Running title (if required): Genetic structuring of a polyembryonous bryozoan

ABSTRACT

Microscale genetic differentiation of sessile organisms can arise from restricted dispersal of sexual propagules, leading to isolation by distance, or from localised cloning. Cyclostome bryozoans offer a possible combination of both: the localised transfer of spermatozoa between mates with limited dispersal of the resulting larvae, in association with the splitting of each sexually produced embryo into many clonal copies (polyembryony). We spatially sampled 157 colonies of *Crisia denticulata* from subtidal rock overhangs from one shore in Devon, England at a geographic scale of c. 0.05 to 130m plus a further 21 colonies from Pembrokeshire, Wales as an outgroup. Analysis of molecular variance (AMOVA) revealed that the majority (67%) of genetic variation was distributed among individuals within single rock overhangs, with only 16% of variation among different overhangs within each shore and 17% of variation between the ingroup and outgroup shores. Despite local genetic variation, pairwise genetic similarity analysed by spatial autocorrelation was greatest at the smallest inter-individual distance we tested (5cm) and remained significant and positive across within-overhang comparisons (<4m). Spatial autocorrelation and AMOVA analyses both indicated that patches of *C. denticulata* located on different rock overhangs tended to be genetically distinct, with the switch from positive to negative autocorrelation, which is often considered to be the distance within which individuals reproduce with their close relatives or the radius of a patch, occurring at the 4-8m distance class. Rerunning analyses with twenty data sets that only included one individual of each multilocus genotype (n=97) or the single data set that contained just the unique genotypes (n=67) revealed that the presence of repeat genotypes had an impact on genetic structuring (PhiPT values were reduced when shared genotypes were removed from the dataset) but that it was not great and only statistically evident at distances between individuals of 1-2m. Comparisons to a further twenty randomisations of the data set that were performed irrespective of genotype (n=97) suggested that this conclusion is not an artefact of reduced sample size. A

resampling procedure using kinship coefficients, implemented by the software package GENCLONE gave broadly similar results but the greater statistical power allowed small but significant impacts of repeat genotypes on genetic structure to be also detected at 0.125-0.5 and 4-16m. Although we predict that a proportion of the repeat multilocus genotypes are shared by
30 chance, such within-overhang distances may represent a common distance of cloned larval dispersal. These results suggests that closely situated potential mates include a significant proportion of the available genetic diversity within a population, making it unlikely that, as previously hypothesised, the potential disadvantage of producing clonal broods through polyembryony is offset by genetic uniformity within the mating neighbourhood.

35 We also report an error in the published primer note of Craig et al. (2001, Molecular Ecology Notes, 1: 281-282): loci Cd5 and Cd6 appear to be the same microsatellite.

INTRODUCTION.

Forces responsible for the evolution and maintenance of sexual reproduction remain enigmatic. The well documented problem is that sex should be prohibitively expensive compared to asexual reproduction. Most attention has been given to obligate sexuals or
40 asexuals, but in many taxa sexual and asexual reproduction coexist (Bell, 1982; Hughes, 1989). The question then becomes one of when and how much sex is optimal and it appears that in many environments the best strategy may be to produce a small fraction of offspring sexually, and the rest asexually (Hurst & Peck, 1996). One intriguing life history strategy that appears to
45 depart from the optimal balance of sexual and asexual reproduction is polyembryony. ‘Polyembryony’ refers to the splitting of a single sexually produced embryo into many clonal copies. By incorporating a sexual phase the various costs of sex seem to be incurred. By then cloning, at least some of the advantages appear forgone. Producing polyembryonic offspring forsakes the genetic diversity inherent within a sexual brood and ‘bets’ on a single unproved
50 genotype—an error equivalent to that of purchasing multiple tickets of the same number in a lottery (Craig et al., 1997). Despite such an apparent handicap, routine polyembryony (as compared to the occasional aberrant production of identical twins) appears to have evolved numerous times, being reported in some rust fungi, algae, higher plants and animals (metazoan phyla include cnidarians, platyhelminths, arthropods, bryozoans, echinoderms and chordates—
55 see Craig et al., 1997; Hughes et al., 2005). More examples of embryonic cloning are being discovered as groups receive sufficient study (e.g. Eaves & Palmer, 2003).

This work focuses on one order of bryozoans, the Cyclostomata (class Stenolaemata), in which all representatives (except the Cinctiporidae, Boardman et al., 1992) are thought to be polyembryonic (Ryland, 1970). Although several circumstances have been suggested that
60 should favour the evolution and maintenance of polyembryony (see Craig et al., 1995; Craig et al., 1997; Giron et al., 2004), most do not seem relevant to sessile organisms like cyclostome bryozoans that brood their offspring to a late stage of development (discussed further in S1.1 of

the ‘Electronic Supplementary Material’ [hereafter ESM]). In this paper we attempt to address a hypothesis for the maintenance of polyembryony in sessile organisms like cyclostome bryozoans made by Ryland (1996). Ryland predicted that the potential for cyclostome bryozoans to produce a genetically diverse sexual brood would be limited within the viscous population structures that would be expected to result from restricted gene flow in populations of colonial marine invertebrates. If all available spermatozoa originate from a similar genetic background the relative loss of offspring diversity caused by polyembryony is reduced.

Investigation of the microgeographic genetic composition of a natural population of a cyclostome bryozoan could test this hypothesis. A finding that mating neighbourhoods were genetically homogeneous would support Ryland’s proposition. Alternatively, evidence that significant genetic variation was distributed among neighbouring sperm donors would go against the hypothesis. As well as helping us to understand Ryland’s (1996) proposition, information on genetic differentiation over small spatial scales may also allow us to quantify the impact of repeat genotypes, which may be the signature of multiple settlement of polyembryonous clonemates, on the genetic structure of the population in order to determine the relative contributions of restricted sperm and larval dispersal and polyembryony to any observed fine-scale genetic structuring.

The ability to use dilute sperm efficiently, and thus the potential for fertilisation at a distance, would have important consequences for gene flow, and therefore genetic structuring, in ‘spermcast’ or ‘egg-brooding free-spawning’ taxa that release sperm into the water but retain their eggs for internal fertilisation. Recent work (Bishop, 1998; Yund, 2000; Pemberton et al., 2003; Johnson & Yund, 2004; Lasker, 2006) published after Ryland’s paper in 1996 has demonstrated that the dynamics of fertilization in spermcast taxa may differ greatly from the externally fertilising models that rely on dispersing clouds of eggs and sperm intersecting during a crucial interval following spawning. Given their ability to accumulate sperm from low concentrations, cross fertilisation, from distances potentially sufficient to maintain genetic

heterogeneity, appears to be obtainable in spermcast animals (Pemberton et al., 2003; Phillippi
90 et al., 2004; but see S1.2 in the ESM for possible risks of extrapolating fertilisation data across
taxa).

Colonial marine invertebrates typically possess non-feeding, lecithotrophic, larvae that
disperse relatively short distances (Jackson, 1986). Direct observation that larvae of the
cyclostome *Tubulipora tuba* were observed to settle on the same piece of kelp as their brood
95 parent at densities ten times greater than on similar substrates only 1m away (McFadden, pers.
com. in Knowlton & Jackson, 1993) and indirect supposition from incidences of colony fusion
of what are assumed to be polyembryonous clonemates of an extant cyclostome (Harmelin,
1974) and an extinct fenestrate relative (McKinney, 1981) support the idea of limited dispersal
in cyclostome larvae. Limited dispersal can lead to genetic structuring of a population as a result
100 of isolation-by-distance, with differentiation possible through genetic drift or local selection.
However despite theoretical expectations of micro-scale genetic structuring (Knowlton &
Jackson, 1993) evidence of this exists for relatively few sessile invertebrates (Yund & O'Neil,
2000). When found population subdivision may (e.g. Hellberg, 1995) or may not (e.g. Burnett et
al., 1995) be a result of the limited dispersal of larvae.

105 As asexual fragmentation and rafting of the adult stage is not expected in cyclostome
bryozoans with the colony form and habitat of the species studied here (discussed in S1.3 in the
ESM), the multiple settlement of genetically identical larvae cloned through polyembryony is
the only route for asexual processes to influence microscale genetic differentiation. A number of
previous studies have investigated the population genetic consequences of cloning at the adult
110 stage in sessile marine invertebrates (e.g. McFadden, 1997 and references therein) and algae
(e.g. Wright et al., 2000; Hämmerli and Reusch, 2003; Alberto et al., 2005). The population
genetic consequences of cloning meiotically produced embryos are less well understood (Engel
et al., 2004).

115 MATERIALS AND METHODS

Bryozoan biology

Bryozoans are aquatic invertebrates that are typically colonial and hermaphroditic, built from replicated zooids budded from a metamorphosed, sexually produced larva. Zooids filter feed with a ciliated, tentacular lophophore. Adjacent zooids are connected, allowing resources to be shared around the colony. Cyclostomes are polymorphic with, amongst others, distinct feeding (auto-) and brooding (gono-) zooids. Spermatogenesis in such species without dedicated male zooids occurs in autozooids, with sperm released into the water column (Silén, 1972). In most bryozoan species eggs are retained and fertilisation is internal. In the class Gymnolaemata sperm are brought into contact with acting female zooids through the feeding current, adhere to tentacles on the lophophore, and enter the maternal coelom (Temkin, 1994). Sperm collection in the Cyclostomata has not been described. Oogenesis in cyclostome bryozoans has been best studied within the genus *Crisia*, where zooidal development is closely linked to the process of branch formation from apical growing points within the erect colony. Adjacent zooids are calcified together into short lengths called internodes, connected by flexible nodes. Many oogonia initially form in the developing internode but most degrade, their host zooids differentiating into autozooids. On some internodes a single (rarely >1) developing zooid differentiates into a gonozooid, on which the lophophore is transitory. The calcareous wall of the developing gonozooid is expanded. Within this brooding space polyembryony occurs when a 'primary embryo' repeatedly buds off clumps of cells that, with slight variation between taxa, go on to develop into independent larvae (Harmer, 1893; Robertson, 1903; Borg, 1926, Ryland, 2000). A brood size of up to 100 embryos has been reported, although in *Crisia* embryos appear to develop, and are presumably released, sequentially such that at any one time the gonozooid contains many fewer mature larvae. Molecular techniques have recently confirmed that embryos within individual gonozooids of *Crisia denticulata* are genetically identical, but that different gonozooids on the same colony may be fertilised by different males (Hughes et al., 2005).

Whether this is the case for all cyclostome species is unknown as other cyclostome families such as the Lichenoporidae or Diastoporidae, which await molecular analysis, have single or fused gonozooids. The Hughes et al. (2005) data found no evidence for self-fertilization. The concurrent retention of genetic diversity between broods is common in non-animal, sessile, polyembryonous taxa such as pines (e.g. Krutovskii & Politov, 1995; Filonova et al., 2002) and red algae (e.g. Engel et al., 2004).

Sample collection and genotyping

Colonies of *Crisia denticulata* were mapped and collected from the underside of rock overhangs in the shallow subtidal by snorkelling at low water springs. Overhangs extended c. 1-6m horizontally along the shore and projected c. 0.5-1.5m from top to bottom as measured along the rock surface. Each overhang, which may have contained one colony to several hundred colonies, was separated horizontally from adjacent overhangs by c. 2-30m. Density of *C. denticulata* across all sampled overhangs was mapped semi-quantitatively within a grid held against the rock wall (5x5cm grid divisions: score 0 = no colonies present; 1 = one or two colonies; 2 = many colonies, >50% cover of *C. denticulata*). The position of each collected colony was recorded from its x,y position within the grid. Colonies were collected semi-randomly to ensure representation from across the range of local densities. Samples from Wembury, near Plymouth, Devon, England (hereafter ‘Wembury’) were collected over the summer of 2001 and are an extension of those first reported by Hughes et al. (2005). Aerial photographs of the study site are provided in S2 in the ESM along with a summary of all mapping, density and genotyping data. Samples from Caerfai Bay, near St Davids, Pembrokeshire, Wales (hereafter ‘Pembrokeshire’, national grid reference SM762241) were collected in July, 2002 from two overhangs located about 20m apart, to serve as an outgroup.

180 colonies were genotyped at six microsatellite loci (Craig et al., 2001, note that all analyses, except that done for linkage disequilibrium, used only five loci—see Results).

Molecular methods duplicated those described for the 2001 samples in Hughes et al. (2005). All PCR products were run at least twice at different concentrations, with PCRs repeated if bands were not clearly sizable. Two colonies from overhang O were dropped from the analysis as no clear product from locus Cd5 could be obtained after three repeat PCRs, giving a final sample size of 178 individuals. Sample size at each overhang is displayed in Table 1.

Genotyping summary statistics

The main focus of this study was the spatial genetic analyses outlined below. To provide a comprehensive data set we also present standard genotype summary measures of linkage disequilibrium (all six loci) and departures from Hardy-Weinberg (HW) expectations (HW exact tests and score tests, locus Cd6 excluded) conducted with the software package GENEPOP (Raymond and Rousset, 1995). Caution is required in the interpretation of some of these results as sample sizes between overhangs varied (range 9-50, see Table 1) and assumptions of HW equilibria are probably unsound given the clonal nature of the mating system.

To compare the observed occurrence of repeat multilocus genotypes with theoretical expectations, probability of identity (PI) was used to calculate the match probability, on the basis of allele frequency data and HW expectations, that two unrelated individuals will share a multilocus genotype by chance. PI and the more conservative PI_{sibs} (see Waits et al., 2001), which estimates the probability of identity between sibs and thus allows for the local genetic structure, were calculated with the software package GENALEX 6 (Peakall and Smouse, 2006). We also assessed P_{gen} , the likelihood that repeat multilocus genotypes were shared by chance using the “round robin” method of Parks and Werth (1993), and P_{sex} , the probability that individuals sharing the same multilocus genotype were derived from a distinct sexual reproductive event, using the software package GENCLONE (Arnaud-Haond and Belkhir, 2007). We also used the permutation methodologies of GENCLONE to visualise whether the

power of the microsatellite markers provided a good estimate of the real number of multilocus genotypes present in the sample (Arnaud-Haond et al., 2005; Gregorius, 2005).

195

Spatial genetic analyses

Data classes: Table 1 shows the five classes of data used in the spatial genetic analyses. Only data set #1, the ‘full Wembury + Pembrokeshire data set’ included animals from the Pembrokeshire outgroup. This outgroup was excluded from spatial autocorrelation analysis because of the vastly different geographic scale of Wembury-Pembrokeshire comparisons. The four remaining Wembury-only data sets were used in all genetic analyses. Data set #2, the ‘full Wembury data set’, lacked the Pembrokeshire animals but was otherwise the same as data set #1. It is equivalent to a ‘ramet level’ analysis as defined by Arnaud-Haond and Belkhir (2007). Data set #2 contained 157 individuals of which 90 shared their multilocus microsatellite genotype with at least one other individual. We were very interested in these repeated genotypes as they may have represented multiple offspring from polyembryonous broods (see Results and Discussion). Removing all 90 repeated genotypes left 67 individuals that contained ‘unique genotypes only’ – this was data set #5. A less constrained method of avoiding repeats was to retain a single representative of each genotype. Owing to the prodigious number of possible combinations of the 90 individuals that shared 30 different microsatellite genotypes, we ran analyses on 20 randomly chosen subsets and called this data set #3 ‘one of each genotype’. This data set is broadly equivalent to the ‘genet level’ resampling approach of Arnaud-Haond and Belkhir (2007). Differences in results between #2 ‘full Wembury data set’ and #3, ‘one of each genotype’ would be expected to result from repeat genotypes and the reduction in sample size ($n = 157$ cf. 97). To better understand the influence of sample size we created a further 20 subsets of $n = 97$, but this time randomly reduced without respect to genotype so that shared microsatellite genotypes remained. This was data set #4 ‘random $n = 97$ ’.

Note that the methodology of randomising the data to generate multiple sets of ‘one of each genotype’ is conservative to situations where sampling is not exhaustive, repeated
220 multilocus genotypes are relatively rare and widespread over the sample area, and/or where there are expectations that the multilocus markers have insufficient power to fully differentiate every clonal individual. This differs from some other methods for differentiating sexual from asexual processes where multiple individuals of the same multilocus genotype are pooled across space to provide a single geographic coordinate and thus a single reduced data set (see e.g.
225 Hämmerli and Reusch, 2003; Alberto et al., 2005) – the ‘genet level’ ‘central coordinates approach’ of Arnaud-Haond and Belkhir (2007).

Software: The main spatial genetic analyses were performed with the software package GENALEX. Pairwise individual by individual genetic distances were calculated with the
230 method fully explained in Smouse and Peakall (1999). This method produces a matrix of squared dissimilarity distances between individuals for each locus. Values can be summed across loci to produce a single multivariate matrix of pairwise, multilocus genetic distances.

Analysis of molecular variance (AMOVA): Individual pairwise genetic distance matrices were
235 analysed by AMOVA (Michalakis and Excoffier, 1996). The main analyses used the multivariate matrix described above. Tests on individual loci were also performed on the ‘full Wembury + Pembrokeshire data set’. Total genetic variation was partitioned into three hierarchical levels: among regions (Wembury vs. Pembrokeshire), populations (rock overhangs) and individuals (single colonies, Peakall et al., 1995). Variation is presented both as the
240 percentage of the total variance and as Φ -statistics (F -statistic analogues): Φ_{PT} is the correlation among individuals within populations; Φ_{PR} the correlation among populations within regions; and Φ_{RT} the correlation among regions. F -statistics derived from the alternative input as a

codominant allelic distance matrix are provided for comparison with the multiallelic, total data set. Statistical significance was determined by random permutation (always set to 1000).

245

Spatial autocorrelation: The main spatial genetic structure analysis used the multivariate matrices described above. This genetic distance matrix was matched as an input to the pairwise geographical distance matrix, calculated as the Euclidean distance between x,y-coordinates. As we were primarily interested in structure at small geographic scales, discrete distance classes were set on an arithmetic doubling scale (with the final distance class extended slightly). The generated autocorrelation coefficient, r , is bounded by -1 and $+1$ and is closely related to Moran's-I. Tests of statistical significance were performed by both random permutation to provide 95% confidence intervals around the null hypothesis of no spatial genetic structure ($r = 0$), and by bootstrapping to estimate the 95% confidence interval about the r value itself (see Peakall et al., 2003). For comparison we repeated analyses using the kinship coefficients of Loiselle et al. (1995) and Ritland (1996). With the software package GENCLONE (Arnaud-Haond and Belkhir, 2007) three additional autocorrelation procedures adapted to the existence of replicate genotypes were performed: (1) using a resampling approach to automatically create and analyse subdatasets with each multilocus genotype represented only once (equivalent to that performed manually in the twenty 'one of each genotype' datasets above) to generate 95% confidence intervals of the influence of multilocus genotypes themselves; (2) using a weighted approach to remove the distances among pairs of identical genotypes from the data set; (3) using central coordinates for each replicated multilocus genotype (fully described by Arnaud-Haond and Belkhir, 2007).

265

Mantel tests: Correlation between the genetic and geographic distance matrices was also analysed by Mantel tests of matrix correspondence (Mantel, 1967; Peakall et al., 1995).

RESULTS

270 **Genotyping summary statistics**

We found identical genotypes at loci Cd5 and Cd6 in 171 out of the 178 *C. denticulata* colonies. This extreme linkage disequilibrium prompted us to look at the Genbank sequences of the original primer note of Craig et al. (2001) which showed the Cd6 clone to be identical to the Cd5 clone at 491 of the 498 bp. Considerable similarity exists in the published primer
275 sequences, with primer overlaps accounting for the 10bp difference in the size of PCR products. We conclude that loci Cd5 and Cd6 are actually the same microsatellite and urge future workers to use one or other, but not both, of the primer pairs. All analysis for this study only used the data from Cd5. With the probably unlikely exception that some of the differences between the Cd5 and Cd6 genotypes are due to indels in the different primer flanking regions this
280 duplication acts as an independent, blind scored, estimate of the genotyping error rate. Assuming it improbable that an identical miscoring error is made in both loci in the same individual the error rate for each locus is half that reported above, so c. 2%. Of the remaining 14 locus pair comparisons linkage disequilibrium may have been detected between loci Cd4 and Cd7 ($\chi^2_{20} = 41.59$, unadjusted $P = 0.003$, sequential Bonferroni 5% $P_{\text{crit}} = 0.0036$) as well as
285 Cd7 and Cd8 ($\chi^2_{20} = 32.90$, unadjusted $P = 0.008$, sequential Bonferroni 5% $P_{\text{crit}} = 0.0038$).

We observed two significant departures from HW equilibrium out of the 66 possible locus x overhang calculations (Cd7 in overhang K and Cd15 in overhang J – fully tabulated in S3.1 in the ESM). Across all overhangs locus Cd7 showed an overall significant departure from HW expectations. Across all loci overhangs J and K both showed an overall significant departure
290 from HW expectations. None of the loci showed significant positive F_{IS} values across all overhangs suggesting that the presence of null alleles was negligible. Score tests (Rousset and Raymond, 1995) for heterozygote deficiencies reported a strong deficit in locus Cd15, which was probably caused by three individuals being incorrectly scored as homozygous for a unique

allele due to a sizing error. Score tests showed that no loci or overhangs had a heterozygote
295 excess.

Permutation methodologies that produced plots of the increase in detected multilocus
genotypes with increasing number of loci did not show an asymptote, suggesting that we
overestimated the true number of repeated multilocus genotypes. Across all colonies the
probability of identity (PI = 0.008), PI_{sibs} (0.110) and P_{gen} (2 colonies $P>0.05$; 48 colonies
300 $P=0.05-0.01$; 21 colonies $P=0.01-0.005$) measures confirmed the relatively low power of the
five microsatellite loci to differentiate genetically distinct individuals, although given the
expected violation of HW principals, caution is required in the exact interpretation of these
results. A slightly more conservative version of one of these tests that takes into account the
estimated F_{IS} in the population (Young et al., 2002), ' $P_{gen}(f)$ ' slightly increased the probabilities
305 (7 colonies $P>0.05$; 54 colonies $P=0.05-0.01$; 21 colonies $P=0.01-0.005$) suggesting that HW
deviations were not conservative to the initial results. With the sample size of 178 individuals
the PI values predict that 1.4 (PI) and 19.5 (PI_{sibs}) individuals would share the same multilocus
genotype by chance alone. This is clearly many fewer than the 90 colonies found in this study
with shared multilocus genotypes and suggests that the multiple settlement and survival of
310 polyembryonous clone mates can be detected but that our measures are imprecise. Similarly the
 P_{gen} and $P_{gen}(f)$ derived binomial measures P_{sex} and $P_{sex}(f)$ suggest that 22 or 23, respectively, of
the 30 distinct multilocus genotypes that were found in more than one colony could statistically
have resulted from sexual rather than asexual processes, although the low power of this test for
the current data set where many repeat genotypes are found only a few times (16 of the 30
315 repeated multilocus genotypes were only found twice) must be considered.

S3.2 and S3.3 in the ESM tabulate allele frequencies for each overhang and sample sizes,
number of alleles, number of effective alleles, information index, observed and expected
heterozygosity and fixation index for each overhang x locus combination.

320 **Spatial genetic analyses**

AMOVA

Table 2 summarises the AMOVA results. Note that the majority of variation is distributed within individual rock overhangs (populations). Table 3 shows the pairwise Φ_{PT} values. The inset of Table 3 shows the multi-dimensional relationship between all populations by Multi-
325 Dimensional-Scaling (software package PRIMER [Clarke & Gorley, 2001]) indicating the lack of differentiation between populations G, H, I and J.

With only the ‘full Wembury data set’, AMOVA revealed a Φ_{PT} of 0.1998, with 80% of the variation distributed within populations and 20% among populations (also shown in Table 2). This Φ_{PT} was greater than any of the 20 ‘one of each genotype’ subsets that had replicate
330 microsatellite genotypes removed (Mean Φ_{PT} = 0.1621, *s.d.* = 0.0090, *n* = 97). In comparison to the ‘full Wembury data set’ more variation was found within populations (range 83-87%) and less among populations (13-17%) in the ‘one of each genotype’. These effects appear not to be due to the reduced sample size, as the ‘random *n* = 97’ subsets showed Φ_{PT} values similar to that of the ‘full Wembury data set’ (mean = 0.1972, *s.d.* = 0.0210), and significantly greater
335 than the ‘one of each genotype’ subsets (Mann-Whitney *W* = 652.5, *p* < 0.0001). The ‘random *n* = 97’ subsets also had variation within (76-83%) and among (17-24%) populations similar to that of the ‘full Wembury data set’. AMOVA of the ‘unique genotypes only’ (*n*=67) exaggerated the trends revealed by the ‘one of each genotype’ subsets with a low Φ_{PT} of 0.1428, and high variation within (86%) rather than between (14%) populations.

340

Spatial Autocorrelation

Figure 1 shows correlograms of pairwise genetic correlation as a function of geographical distance for the four Wembury data sets (#2-5 in Table 1). All within-overhang pairwise geographic distances were less than 4m. The 1-2m distance class also contained some of the
345 between-overhang comparisons of overhangs L and M (hereafter LM). The 2-4m class

contained the remainder of LM as well as some of NN and JJ. The 4-8m class held only GH and HI, the 8-16m class only GI and JK. The 16-32m class contained KL, KM, LN, and MN. The 32-64m class comprised GJ, GK, HJ, HK, IJ, IK, JL, JM, JN, KM and NO (fully tabulated in S4 of the ESM).

350 All four data sets had the highest positive r values at the smallest distance class of ≤ 0.125 m. Most values remained positive and significant for distances up to 1m. Only in the ‘full Wembury data set’ did r values remain consistently positive and significant up to 4m, although this trend was evident in many of the ‘random $n = 97$ ’ runs. The vast majority of the ‘one of each genotype’ runs had r values around zero in the 1-2m distance class although most runs
355 regained positive, significant autocorrelation for the 2-4m class. Even though repeat genotypes made up a greater proportion of pairwise comparisons at other within-overhang spatial scales (Figure 1A, sample size data) the test statistic of genetic similarity changed slightly at 0.125-0.25m and considerably at the 1-2m distance class. At intermediate distances between 4 and 32m autocorrelation decayed such that all data sets displayed significant negative
360 autocorrelation at the 16-32m class. In the between-overhang comparisons of 4-16m, omission of repeat genotypes slightly increased genetic similarity. This counterintuitive result likely stems from the dominance of comparisons between overhangs G, H and I at these spatial scales. G, H and I were found to be genetically uniform by AMOVA (Table 3) and are composed of a relatively low proportion of repeated genotypes (33% compared to 66% in the remaining
365 overhangs combined). All data sets showed autocorrelation around zero at 32-64m, probably due to the clustering of genetically uniform overhangs GJ, HJ and IK into otherwise dissimilar pairwise comparisons. Significantly negative autocorrelation was always seen for the largest distance class (64-150m). Repeating analyses using the kinship coefficients of Loiselle et al. (1995) and Ritland (1996) and the programme GENCLONE gave broadly similar results for the
370 ‘Full Wembury data set’ (= ‘ramet level’ analysis, Figure 2). The greater statistical power of GENCLONE’s resampling approach allowed small but significant impacts of repeat genotypes

on genetic structuring to be also detected at 0.125-0.5, 1-2 and 4-16m (Figure 2) that were in some instances not so apparent in the main analyses shown in Figure 1. Using GENCLONE's weighted approach to remove the distances among pairs of identical genotypes from the data set allows for an estimation of the clonal subrange, the distance beyond which repeat multilocus genotypes have negligible effects on genetic structure as less than 1% of pairs are of the same genotype (Alberto et al., 2005). As shown in S5 of the ESM this distance class, where correlograms merge, is at 8-16m, a distance that represents the lower end of the inter-overhang comparisons. As predicted in the Material and Methods section, using central coordinates for each replicated multilocus genotype gave a pattern that fluctuated wildly and is difficult to interpret (S5 of the ESM).

As the ability to detect nonrandom genetic structure is influenced by the distance class size chosen, we also analysed increasing cumulative distance size classes from the minimum resolution used in this study of 0-0.05m up to 0-120m (maximum individual to individual distance in the data = 133m). Thus the analysis displayed in Figures 1 and 2 used discrete distance classes (e.g. 0-1, 1-2, 2-3), whereas the second analysis, displayed in S6 of the ESM, used cumulative distance classes (e.g. 0-1, 0-2, 0-3). The highest value of r was found at the smallest distance class of 0-0.05m. As cumulative distance increased the r value dropped, indicating a loss of resolution. Negative autocorrelation at greatest pairwise geographic distances (against which the statistical comparison would be made), combined with strong positive autocorrelation at the intra-overhang scale, helped r values remain positive at large cumulative distances.

Mantel tests

Results with the 'full Wembury + Pembrokeshire data set' ($r_{xy} = 0.137, p = 0.007$) and the 'full Wembury data set' ($r_{xy} = 0.073, p = 0.021$) corroborated spatial autocorrelation data in indicating that genetic structuring was present. When sample sizes were reduced in data sets #3-

5, however, the less powerful Mantel tests generally failed to detect structuring. The lack of resolution of the Mantel tests probably results from a more complex relationship between geographic and genetic distances than the linear assumptions of Mantel procedures (as
400 represented by the inconsistent slope of the correlograms in Figures 1 and 2, full results in S7 of the ESM).

DISCUSSION AND CONCLUSIONS

405 The discrete distribution of *Crisia denticulata* made it possible to define individual rock overhangs as testable subpopulations *a priori*. In doing so AMOVA showed significant differentiation between most rock overhangs separated by only a few meters. Spatial autocorrelation statistics, which do not require subpopulations to be defined *a priori*, also revealed significant genetic dissimilarity at scales representing between-overhang pairwise
410 comparisons, with genetic similarity found within overhangs. Spatial autocorrelation techniques have been used extensively by botanists (e.g. Hämmerli and Reusch, 2003; Vekemans and Hardy, 2004; Migliaccio et al., 2005), but have limited history in studies of sessile marine animals where results have (Yund & O'Neil, 2000) or have not (McFadden & Aydin 1996) found moderately small-scale genetic structuring. The 4-8m distance class at which spatial
415 autocorrelation changed from being positive to negative, which represents the extent of a genetically similar patch, is remarkably similar to the 5.4-7.2m distance class described by Yund & O'Neil (2000) in a population of the non-polyembryonous, spermcast colonial ascidian *Botryllus schlosseri*. Spatial autocorrelation statistics are particularly useful for the analysis of small-scale genetic structure because they allow structure to be detected down to the minimum
420 distance between sampled individuals. In our study fine scale analysis revealed the strongest genetic similarity at the smallest pairwise distances (same or adjacent 5x5cm quadrat division), suggesting restricted gene flow within overhangs that would not have been detected by

traditional methods. Such a result suggests small-scale dispersal of sperm and/or larvae in this species.

425 By analysing data with and without putative multiple representatives of individual clones, components of genetic structure attributable to asexual processes can be separated from the effects of isolation-by-distance during sexual reproduction. In this study the strength of within-overhang genetic similarity was increased by a small but significant amount by the presence of repeat multilocus genotypes at the distance classes of 0.125-0.5, 1-2 and 4-16m. As

430 demonstrated by the PI tests, permutation methodologies, P_{gen} and P_{sex} tests repeat genotypes are expected even in the absence of cloning, their probability being a function of relatedness and the frequency distribution of alleles at the loci studied. Although conservative to the conclusions drawn, it is almost certain that a proportion of the repeated genotypes in our data were not polyembryonous clone mates. Thus, although 0.125-0.5, 1-2 and 4-16m may represent

435 statistically prominent distances of cloned larval dispersal, we cannot exclude the possibility that some repeat genotypes are genetically similar individuals (e.g. full- or half-siblings) that our markers cannot distinguish rather than polyembryonous clonemates. In that case these distances could reflect the scale of non-clonal larval dispersal (same maternal colony but different gonozooids) and perhaps the movement of fertilising sperm. Direct tests of sperm and

440 larval transport under field conditions would be needed to investigate this (e.g. Levin, 1990). Our findings, that embryonic cloning makes a detectable (Φ_{PT} values were reduced when shared genotypes were removed from the dataset) but minor contribution to the genetic structuring of a sessile polyembryonous species, is consistent with previous reports from a terrestrial pine (Rogers et al., 1999) and a red alga (Engel et al., 2004, where repeated diploid genotypes could

445 also result from independent fertilisations between haploid parents, further weakening the role that polyembryony plays in determining the genetic structure). The genetic footprint of this form of polyembryony therefore differs greatly from the vegetative spread through rhizomes or plant fragments reported for clonal marine plants where autocorrelation studies must explicitly

consider the fact that individual clones can dominate large areas (e.g. Montalvo et al., 1997;
450 Reusch et al., 1999; Hämmerli and Reusch, 2003).

Despite the finding of genetic similarity at small spatial scales, AMOVA reported the majority of genetic variation (67 or 80% depending on the data class used) to be distributed between colonies within individual rock overhangs. Similar findings have been reported for a polyembryonous intertidal red alga (Engel et al., 2004). Despite finding that the spatially closest
455 individuals had the highest pairwise genetic similarity such results suggest that closely situated potential mates contain a significant proportion of the available genetic diversity within a population. This runs counter to Ryland's (1996) suggested explanation of polyembryony in cyclostome bryozoans that the relative loss of offspring genetic diversity caused by polyembryony is reduced in viscous, locally homogeneous, populations. What exactly
460 constitutes 'sufficient' local genetic variation to favour a purely sexual strategy over the polyembryonous alternative is, however, difficult to know, which is a weakness of this study and a challenge for the testing of Ryland's hypothesis itself. It is clear that we are a long way from being able to quantify the precise costs of polyembryony against the alternatives of a purely sexual or asexual mode of reproduction in species such as cyclostome bryozoans. Given
465 the complexity of the focused attempts over the last few decades to quantify the relative costs of apparently 'simple' sexual versus asexual reproduction (e.g. Bell, 1982; Goddard et al., 2005) this task seems daunting.

Larval and post-settlement mortality are thought to be the main factors limiting population growth in most marine organisms (e.g. Morgan, 1995), although studies of brooded
470 lecithotrophic larvae, such as found in the Bryozoa, are limited. If much of a mother's fitness depends on the sheer number of larvae that she is able to produce and larval mortality occurs randomly with respect to larval genotype (but see Schmidt & Rand, 1999), then the apparent costs of polyembryony may be invisible to selection. Genetic diversity of a female's offspring may be favoured, however, if genetic diversity partially reduces brood-wide larval mortality by

475 increasing the chance of genotype-environmental matching (the ‘tangled bank’ hypothesis of
Bell, 1982). Although polyembryony in *C. denticulata* forces genetic uniformity within a single
brood, some genetic diversity is maintained by the presence of multiple broods (Hughes et al.,
2005). This diversity may be sufficient to accrue much of the larval survival benefit. Further, the
sequential release of genetically uniform embryos over an extended time period by cyclostomes
480 and red algae seems to allow one larval genotype to be repeatedly tested against a variety of
environments as conditions change over the time period of brood release. Such testing of a few
genotypes against a changing environment may differ very little from regular sexual
reproduction that tests many genotypes against an environment at once. This type of
polyembryony that spreads the cloned genet across space and time with a concurrent retention
485 of genetic diversity between broods perhaps seems less of a paradox than initially thought.

A summary of the different hypotheses, tests and results of this study are tabulated as S8
of the ESM.

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Figure legends

Figure 1. Correlograms showing the pairwise genetic correlation (r , solid line) as a function of
discrete geographic distance (x-axis, value represents the upper end of the range). Parts A-D
650 represent the four data classes of Wembury individuals. Only A and C contain repeat
microsatellite genotypes (see Methods and Table 1). Dashed lines show the 95% confidence
interval around the null hypothesis of a random distribution of genotypes as generated by
permutation. Error bars are the 95% confidence interval about r as estimated by bootstrapping

(not shown in B and C for clarity). Numbers of paired individuals at each distance class are
655 provided just above the x-axis. Value in parenthesis in part A is the number of comparisons
between repeat genotypes.

Figure 2. Correlograms generated using the kinship coefficients of Loiselle et al. (1995 – upper
figure) and Ritland (1996 – lower figure). Solid lines represent results for the ‘Full Wembury
660 data set’ (= ‘ramet level’ analysis). Dashed lines show the 95% confidence intervals generated
by the resampling approach of GENCLONE that shows structure in the absence of multilocus
genotypes.

Table 1. Summary of the five data sets used in the genetic analyses. Values represent numbers of individual colonies within each overhang, totaled in the right hand column. The range of values shown in data sets #3 and #4 reflects differences between randomisations. ‘Ng:N’ values represent the proportion of unique multilocus genotypes to the number of individuals in each overhang.

#	Overhang ID	Wembury									Pembrokeshire		n
		G	H	I	J	K	L	M	N	O	Pem Y	Pem Z	
1	Full Wembury + Pembrokeshire data set	12	9	19	50	15	9	10	18	15	14	7	178
2	Full Wembury data set	12	9	19	50	15	9	10	18	15	-	-	157
3	One of each genotype	9-12	6-9	13-17	21-25	8	6-7	7-9	12-14	7-11	-	-	97
4	Random $n = 97$	4-9	3-8	10-15	26-35	6-12	3-9	4-9	9-13	7-12	-	-	97
5	Unique genotypes only	9	6	12	10	5	5	6	8	6	-	-	67
	Ng:N	0.75	0.66	0.63	0.20	0.33	0.56	0.60	0.44	0.40			

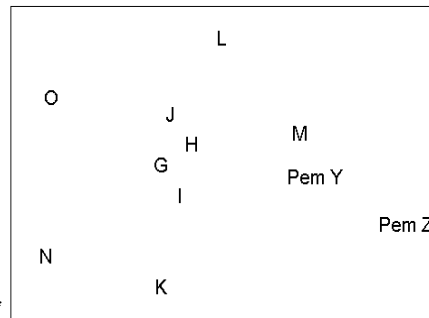
Table 2. Results of AMOVA showing Phi values and the percentage of variation apportioned among regions (Wembury versus Pembrokeshire), populations (rock overhangs) and individuals for individual microsatellite loci and the multivariate total in the “Full Wembury + Pembrokeshire data set”, $n = 178$. p values represented by asterisks = * 0.050 – 0.011, ** 0.010 – 0.006, *** 0.005 – 0.001. Figures in parenthesis are equivalent values for the alternative input as codominant allelic distance matrix for calculation of F_{ST} . Figures in square brackets show the percentage of variation apportioned among populations and individuals in the “Full Wembury data set”, $n = 157$ to show the similarity of signal in all five loci when Pembrokeshire samples are removed from the analysis.

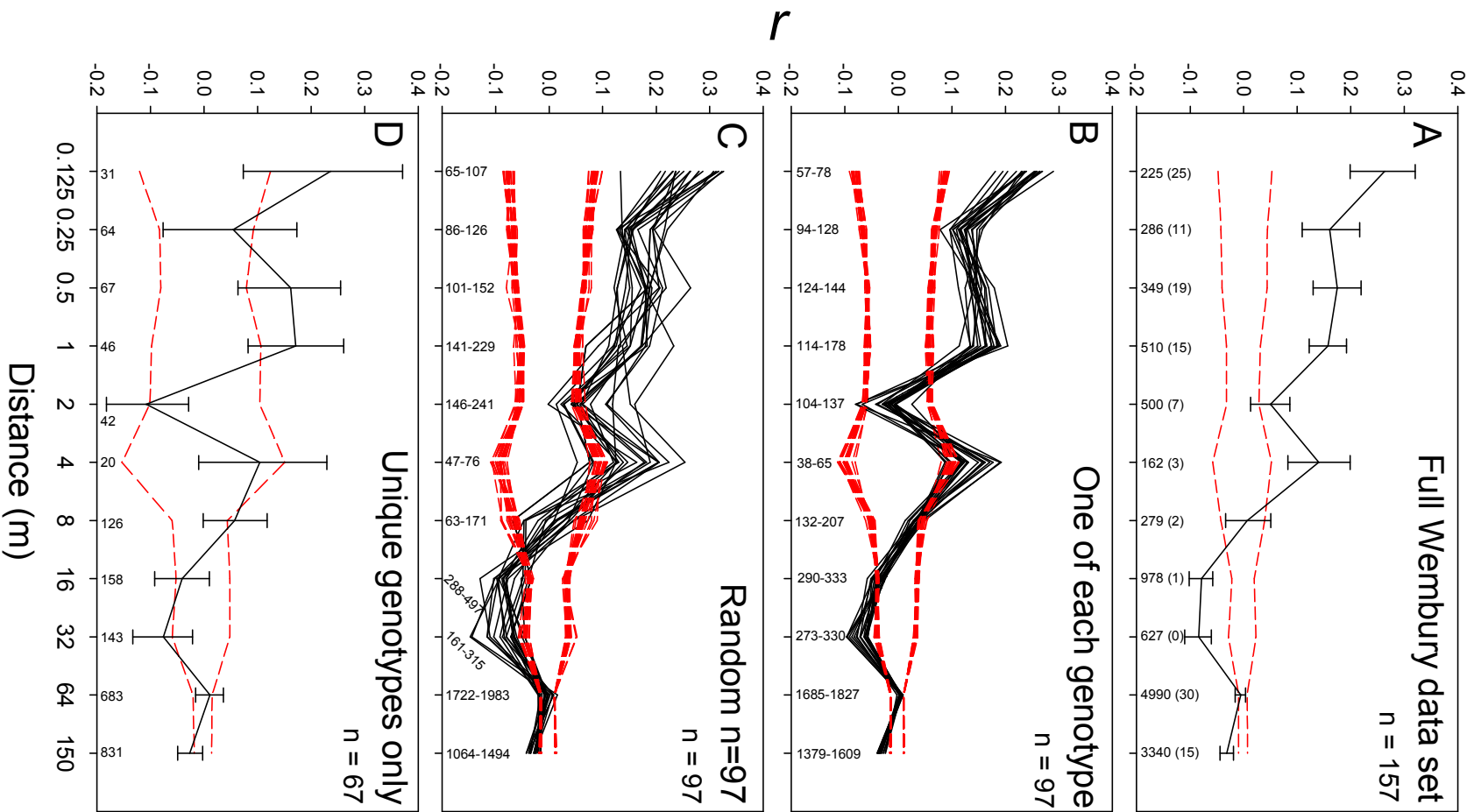
	Total	Cd4	Cd5	Cd7	Cd8	Cd15
<i>n</i> Alleles	25	2	5	10	3	5
	%	%	%	%	%	%
Among regions	17 (10)	0	0	41	2	0
Among pops./regions	16 (10) [20]	22 [25]	21 [24]	10 [16]	8 [9]	28 [29]
Indiv./within pops	67 (80) [80]	78 [75]	79 [76]	49 [84]	90 [91]	72 [71]
Φ_{RT}	0.166 ($F_{rt} = 0.010$)***	-0.102	-0.107	0.407***	0.016	-0.123
Φ_{PR}	0.189 ($F_{sr} = 0.106$)***	0.223***	0.209***	0.163***	0.086**	0.270***
Φ_{PT}	0.323 ($F_{st} = 0.195$)***	0.143*	0.125*	0.504***	0.010*	0.180***

680 Table 3. Pairwise Φ_{PT} values plus significance asterisks (see Table 2) of full data set. ‘G’ to ‘O’ are the individual Wembury overhangs, ‘Pem Y’ and ‘Pem Z’ are the two overhangs of the Pembrokeshire outgroup. Some caution is required in interpreting these pairwise overhang comparisons as sample size varied as described in Table 1. Inset shows an MDS plot of this data set, stress = 0.09. S2B of the ESM provides an aerial photograph of the study site to show the

685 geographic relationship between these overhangs.

G																				
H	0.012																			
I	0.000	0.032																		
J	0.056	0.022	0.062*																	
K	0.123*	0.228***	0.059	0.281***																
L	0.112*	0.175*	0.236***	0.244***	0.295***															
M	0.133*	0.157***	0.210***	0.208***	0.313***	0.120*														
N	0.115*	0.321***	0.176***	0.321***	0.239***	0.379***	0.329***													
O	0.064	0.263***	0.208***	0.247***	0.287***	0.259***	0.335***	0.250***												
Pem Y	0.202**	0.202***	0.195***	0.223***	0.291***	0.233***	0.047	0.373***	0.418***											
Pem Z	0.353**	0.410***	0.291***	0.340***	0.359***	0.431***	0.229***	0.491***	0.554***	0.000										
	G	H	I	J	K	L	M	N	O	Pem Y										





690

Figure 1

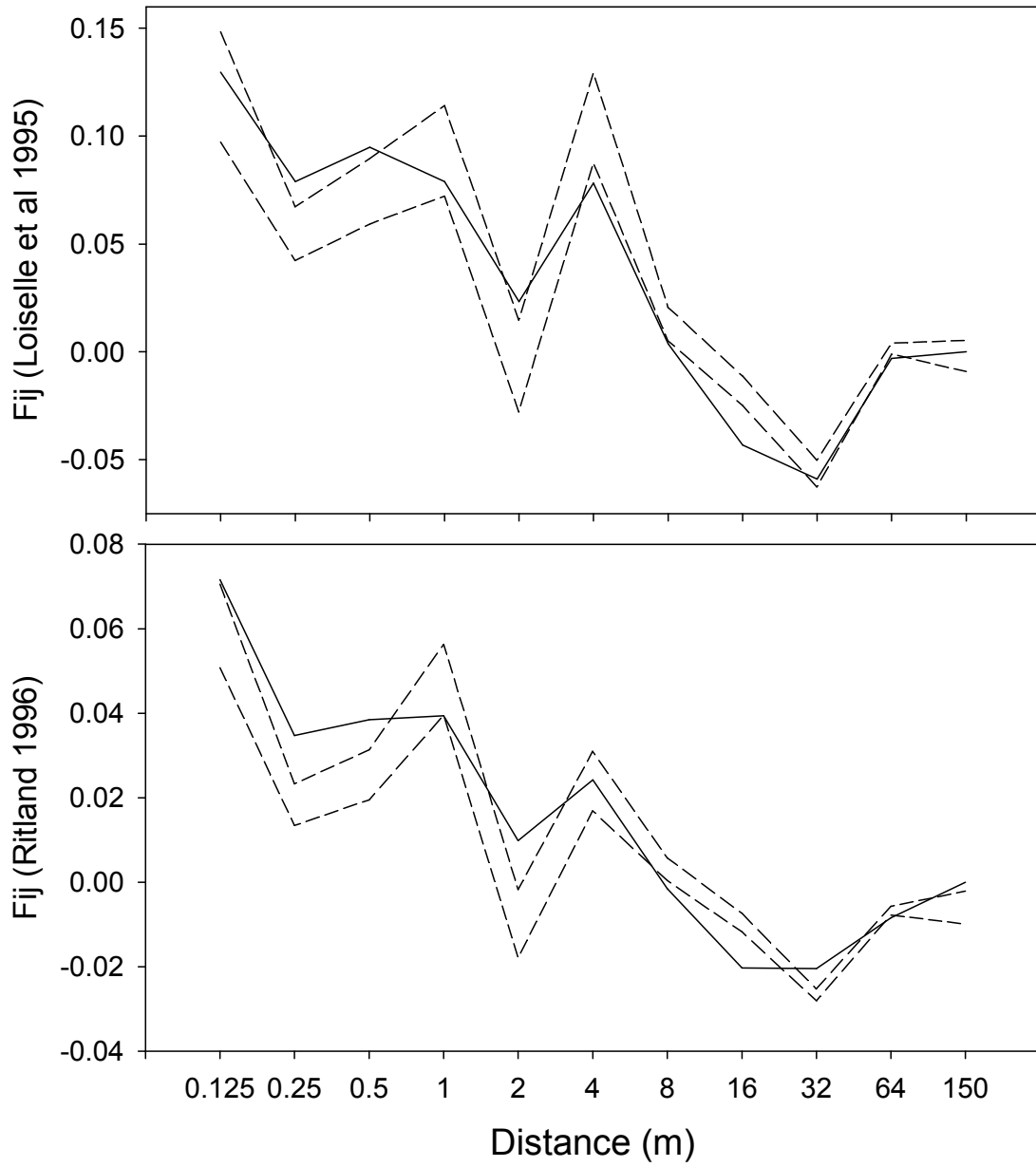


Figure 2

Electronic supplementary material

695

S1. Considerations arising from the biology of *Crisia denticulata*

S1.1. The relevance to cyclostome bryozoans of mainstream hypotheses for the evolution and maintenance of polyembryony.

700 Several, non-exclusive circumstances have been suggested that should favour the evolution and maintenance of polyembryony in animals. In environments of variable quality and where the mother and developing offspring are separated, the offspring may have more information regarding optimal clutch size than the mother. It may be selectively advantageous to limit initial investment and allow polyembryony to facultatively increase brood size (Craig et al., 1995). For
705 organisms like cyclostome bryozoans that brood their offspring to a late stage of development, away from direct environmental cues, this does not seem pertinent. Information available to the female after oviposition, nevertheless, may still be relevant. By avoiding the early stages of reproduction once embryonic budding has been initiated, polyembryony might allow relatively rapid adjustment of larval output, perhaps in response to non-predictable controls such as
710 planktonic food abundance (Craig et al., 1997).

A further possible advantage of polyembryony to the mother's fitness is that by ensuring genetic uniformity within broods, sibling rivalry becomes non-adaptive, and sibling co-operation more likely (Giron et al., 2004). This is probably of importance to parasitoid species where most of the cost of offspring provisioning is borne by the host and direct maternal control is not
715 possible. In brooding species like *Crisia denticulata*, however, the continued existence of competing evolutionary interests should ensure tight maternal control of resources.

S1.2. The likelihood of sperm limitation in cyclostome bryozoans.

720 Caution may be required in extrapolating fertilisation data across taxa as direct tests have not
been performed on any cyclostome species. Cyclostomes have a relatively simple form of filter
feeding (Nielsen & Riisgård, 1998) which, when combined with the as yet unknown
conformation of released sperm, may have significance for efficiency of sperm capture and
avoidance of sperm ingestion (J. S. Ryland, pers. com.) and some species are found at low
density (Hayward & Ryland, 1985; J. S. Ryland, pers. com.).

725

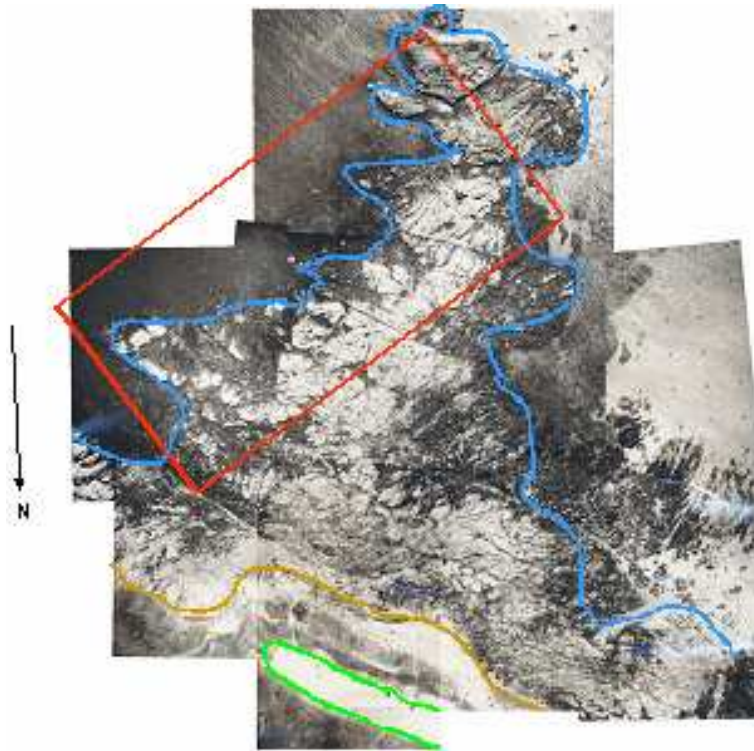
S1.3. Asexual fragmentation is not expected in cyclostome bryozoans like *Crisia*

denticulata. Certain 'sessile' aquatic invertebrates possess limited powers of locomotion, which
when combined with colony fission can shuffle genetic individuals across a landscape (e.g.
Ryland et al., 1984). At slightly greater spatial scales colony fission, detachment from the
730 substrate and subsequent reattachment can move clonal fragments away from the original
settlement location (e.g. Bavestrello et al., 2000). In addition, fouling of natural (e.g. algae,
pumice) or anthropogenic (e.g. ships, plastics) floating substrates can significantly influence
gene flow by moving adults over potentially great distances (Watts et al., 1998). We are
unaware of any evidence for locomotion or colony fission in cyclostome bryozoans. In *C.*
735 *denticulata* a flexible and erect growth form and habitat preference for rock overhangs would
seem to greatly reduce the probability of successful asexual dispersal of the adult stage. Note
that in the absence of locomotion or detachment-reattachment, colony fission alone could not
account for the genetic similarities at scales of 0.125-0.5 and 1-2m that are of interest to
discussions of polyembryony, as the diameter of the single holdfast of the small, bushy colonies
740 is only c. 0.5 to 1.5cm.

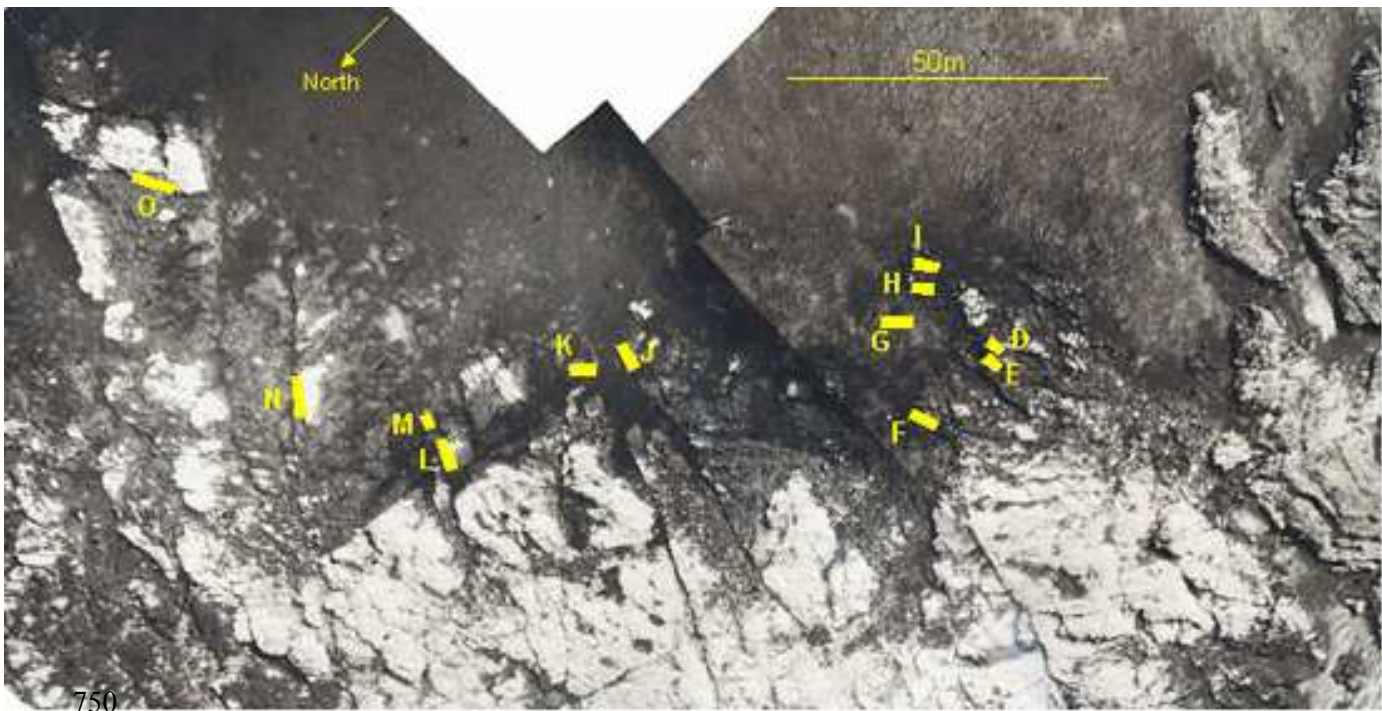
S2. Aerial photographs and summary of mapping and genotyping

S2A. Composite aerial photograph of the Wembury study site. Brown line marks cliff edge, green line is edge of car park. Blue line indicates the approximate extent of rock exposed at low tide. Red box is enlarged in B.

745



S2B. Location of individual overhangs. D, E and F contained isolated colonies of *C. denticulata* and do not form part of the current study.

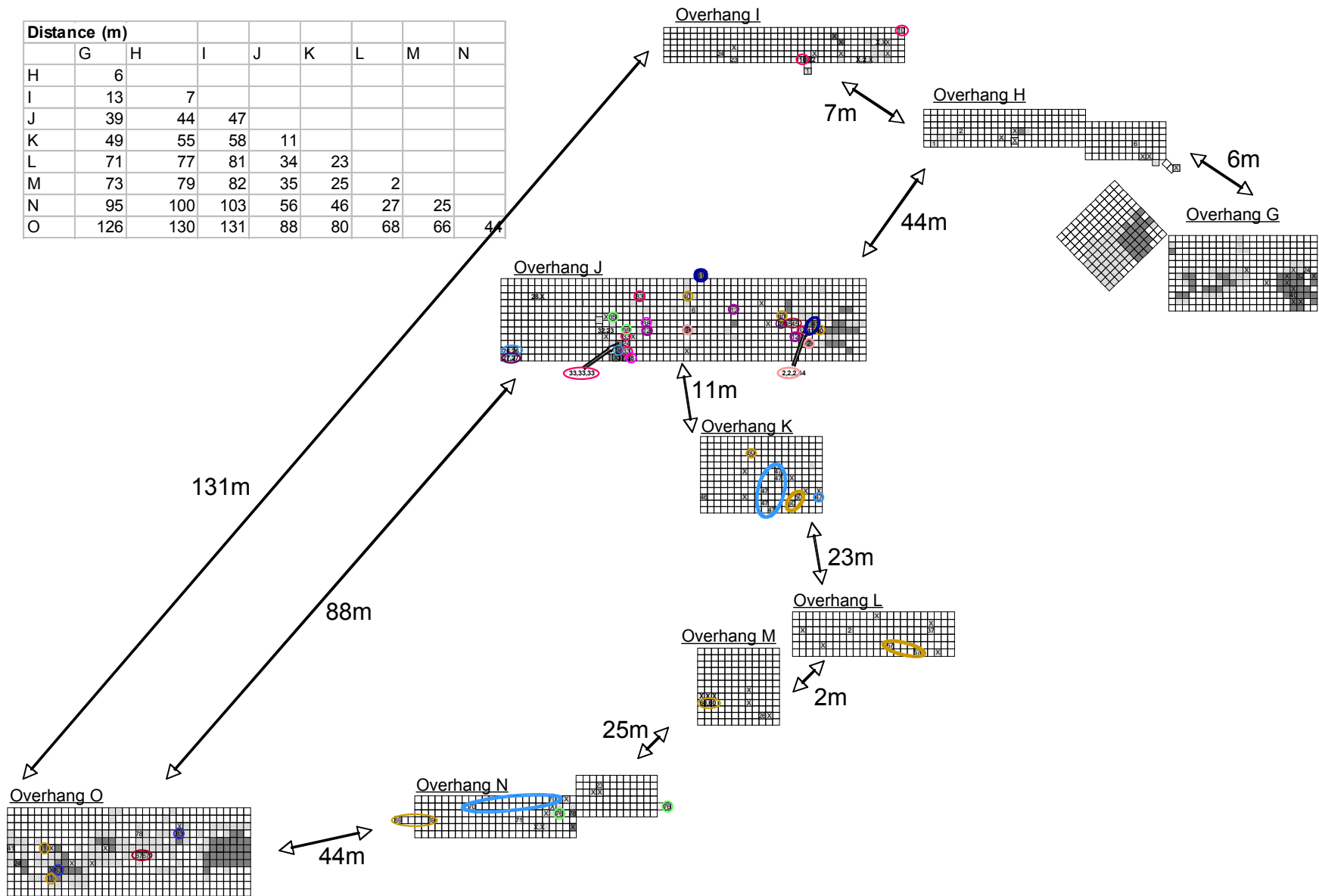


S2C. Graphical summary of mapping, density and genotyping data.

755 Pairwise distances between all overhangs are boxed, with a subset of key distances drawn on the diagram to emphasise the non-linear scale. The small squares within each overhang represent the 5x5cm sampling grids. A 5x5cm grid with no shading contained no *C. denticulata* colonies. A 5x5cm grid with light grey shading was scored as '1' (= one or two colonies), and with dark grey shading as '2' (= many colonies, >50% cover of *C. denticulata*). The spatial distribution of *C. denticulata* can be seen to be clumped.

760 An 'x' shows the location of a colony with a unique microsatellite genotype. Numbers represent genotypes for which >1 colony was sampled. Data from individual 5x5cm grids from which multiple colonies were genotyped is shown by several numbers or x's separated by a comma. When there was insufficient room the data is connected to the 5x5cm grid by an arrow. Repeat genotypes from single overhangs are colour coded (note colours are not consistent between
765 different overhangs).

Distance (m)								
	G	H	I	J	K	L	M	N
H	6							
I	13	7						
J	39	44	47					
K	49	55	58	11				
L	71	77	81	34	23			
M	73	79	82	35	25	2		
N	95	100	103	56	46	27	25	
O	126	130	131	88	80	68	66	44



S3. Genotyping summary statistics.

770 S3.1. Table of test results for departures from Hardy-Weinberg equilibrium.

Unadjusted P -values of exact tests using the Markov chain method (above) and F_{IS} (Weir and Cockerham 1984) (below, italics in main body of table only) for all locus x overhang combinations, all loci across overhangs (right hand column), all overhangs across loci (bottom row) and all data combined (emboldened, bottom right hand cell). Unadjusted P -

775 values of score tests (Rousset and Raymond, 1995) for a heterozygote deficit across loci and overhangs are shown in square brackets. Significant P -values at the 5% level after sequential Bonferroni corrections are marked with an asterisk. Numbers in parenthesis represent sample size for each overhang or number of alleles at each locus. Dashes show locus x overhang combinations with insufficient polymorphism for calculations. Table
780 S3.2 provides allelic frequencies for each overhang that may help interpreting this data.

Overhang ID (sample size)	Locus (no. alleles)					All loci
	Cd4 (2)	Cd5 (5)	Cd7 (10)	Cd8 (3)	Cd15 (5)	
G (12)	0.2900 <i>0.353</i>	1 <i>-0.158</i>	0.4503 <i>-0.267</i>	0.2545 <i>0.436</i>	1 <i>-0.0114</i>	0.7515 [0.5237]
H (9)	1 <i>-0.333</i>	- -	0.5438 <i>0.165</i>	- -	- -	0.8797 [0.4720]
I (19)	0.3558 <i>0.288</i>	- -	0.7649 <i>0.098</i>	1 <i>-0.059</i>	1 <i>-0.067</i>	0.9569 [0.2226]
J (50)	0.0460 <i>-0.307</i>	1 <i>-0.029</i>	0.7923 <i>0.07</i>	0.7794 <i>-0.119</i>	0.0004* <i>1</i>	0.0102 [0.0617]
K (15)	1 <i>0.103</i>	0.0193 <i>0.774</i>	0.0001* <i>-0.092</i>	0.2373 <i>-0.4</i>	1 <i>-0.037</i>	0.0016* [0.3830]
L (9)	1 <i>0.226</i>	0.7253 <i>-0.157</i>	0.3995 <i>-0.153</i>	- -	- -	0.8652 [0.6395]
M (10)	1 <i>-0.2</i>	0.4798 <i>0.386</i>	0.0840 <i>0.229</i>	- -	0.0719 <i>0.449</i>	0.1676 [0.0157]

N (18)	0.6458 <i>-0.141</i>	- -	0.5984 <i>-0.071</i>	0.5294 <i>-0.259</i>	1 <i>-0.045</i>	0.9212 [0.9203]
O (15)	0.1419 <i>-0.439</i>	0.1017 <i>0.14</i>	0.1423 <i>0.344</i>	1 <i>-0.12</i>	- -	0.1382 [0.0500]
PemY (14)	0.5545 <i>0.217</i>	0.2193 <i>0.447</i>	0.1024 <i>0.182</i>	1 <i>-0.04</i>	0.0891 <i>0.35</i>	0.1788 [0.0137]
PemZ (7)	0.4398 <i>0.478</i>	- -	- -	1 <i>-0.091</i>	- -	0.8023 [0.3450]
All overhangs	0.6877 [0.7503]	0.2249 [0.0236]	0.0083* [0.3566]	0.9651 [0.8980]	0.0269 [0.0000*]	0.000093 [0.0096]

Table S3.2 Allelic frequencies for each overhang.

Locus Allele	Cd4			Cd5							Cd7							Cd8				Cd15			
	154	164	179	181	183	188	189	148	149	150	151	152	162	170	172	174	178	146	148	152	126	128	130	132	133
G	0.417	0.583	0	0.833	0	0	0.167	0.458	0	0.042	0	0.250	0.042	0.125	0.083	0	0	0.167	0.833	0	0	0.125	0.042	0.833	0
H	0.278	0.722	0	0.944	0	0	0.056	0.278	0.056	0	0.056	0.389	0	0.056	0.111	0.056	0	0.056	0.944	0	0	0	0	1.000	0
I	0.500	0.500	0	0.974	0	0	0.026	0.289	0	0	0	0.368	0	0.079	0.184	0.053	0.026	0.263	0.737	0	0	0.079	0.026	0.895	0
J	0.240	0.760	0	0.950	0	0	0.050	0.400	0	0.020	0	0.310	0	0.020	0.250	0	0	0.250	0.740	0.010	0	0	0	0.960	0.040
K	0.767	0.233	0	0.833	0	0	0.167	0.133	0	0.067	0	0.267	0	0.100	0.133	0.200	0.100	0.300	0.700	0	0	0.067	0	0.933	0
L	0.278	0.722	0	0.500	0.056	0	0.444	0.278	0	0.333	0	0.222	0	0	0.167	0	0	0	1.000	0	0	0.056	0	0.944	0
M	0.200	0.800	0	0.750	0	0	0.250	0.200	0	0.050	0	0.050	0.050	0	0.450	0.100	0.100	0	1.000	0	0	0	0.250	0.650	0.100
N	0.611	0.389	0.028	0.972	0	0	0	0.583	0	0.139	0	0.056	0	0.167	0.056	0	0	0.222	0.778	0	0	0.417	0.083	0.500	0
O	0.500	0.500	0	0.700	0	0.033	0.267	0.833	0	0	0	0.100	0	0.067	0	0	0	0.133	0.867	0	0	0.033	0	0.967	0
All Pem	0.357	0.643	0	0.881	0	0	0.119	0.024	0	0.024	0	0.071	0	0.048	0.833	0	0	0.095	0.905	0	0.024	0.048	0.048	0.881	0

785

S3.3. Sample sizes (n), number of alleles (Na), number of effective alleles (Ne),

information index (I), observed (Ho) and expected (He) heterozygosity and fixation

790 index (F) for each overhang x locus combination.

Overhang	Locus	n	Na	Ne	I	Ho	He	F
G	Cd4	12	2	1.946	0.679	0.333	0.486	0.314
	Cd5		2	1.385	0.451	0.333	0.278	-0.200
	Cd7		6	3.349	1.436	0.917	0.701	-0.307
	Cd8		2	1.385	0.451	0.167	0.278	0.400
	Cd15		3	1.405	0.544	0.333	0.288	-0.157
H	Cd4	9	2	1.670	0.591	0.556	0.401	-0.385
	Cd5		2	1.117	0.215	0.111	0.105	-0.059
	Cd7		7	3.951	1.610	0.667	0.747	0.107
	Cd8		2	1.117	0.215	0.111	0.105	-0.059
	Cd15		1	1.000	0.000	0.000	0.000	N/A
I	Cd4	19	2	2.000	0.693	0.368	0.500	0.263
	Cd5		2	1.054	0.122	0.053	0.051	-0.027
	Cd7		6	3.800	1.490	0.684	0.737	0.071
	Cd8		2	1.633	0.576	0.421	0.388	-0.086
	Cd15		3	1.238	0.396	0.211	0.193	-0.094
J	Cd4	50	2	1.574	0.551	0.480	0.365	-0.316
	Cd5		2	1.105	0.199	0.100	0.095	-0.053
	Cd7		5	3.131	1.233	0.640	0.681	0.060
	Cd8		3	1.639	0.615	0.440	0.390	-0.129
	Cd15		2	1.083	0.168	0.000	0.077	1.000
K	Cd4	15	2	1.557	0.543	0.333	0.358	0.068
	Cd5		2	1.385	0.451	0.067	0.278	0.760
	Cd7		7	5.844	1.853	0.933	0.829	-0.126
	Cd8		2	1.724	0.611	0.600	0.420	-0.429
	Cd15		2	1.142	0.245	0.133	0.124	-0.071
L	Cd4	9	2	1.670	0.591	0.333	0.401	0.169
	Cd5		3	2.219	0.868	0.667	0.549	-0.213
	Cd7		4	3.767	1.355	0.889	0.735	-0.210
	Cd8		1	1.000	0.000	0.000	0.000	N/A
	Cd15		2	1.117	0.215	0.111	0.105	-0.059
M	Cd4	10	2	1.471	0.500	0.400	0.320	-0.250
	Cd5		2	1.600	0.562	0.500	0.375	-0.333
	Cd7		7	3.704	1.591	0.600	0.730	0.178
	Cd8		1	1.000	0.000	0.000	0.000	N/A

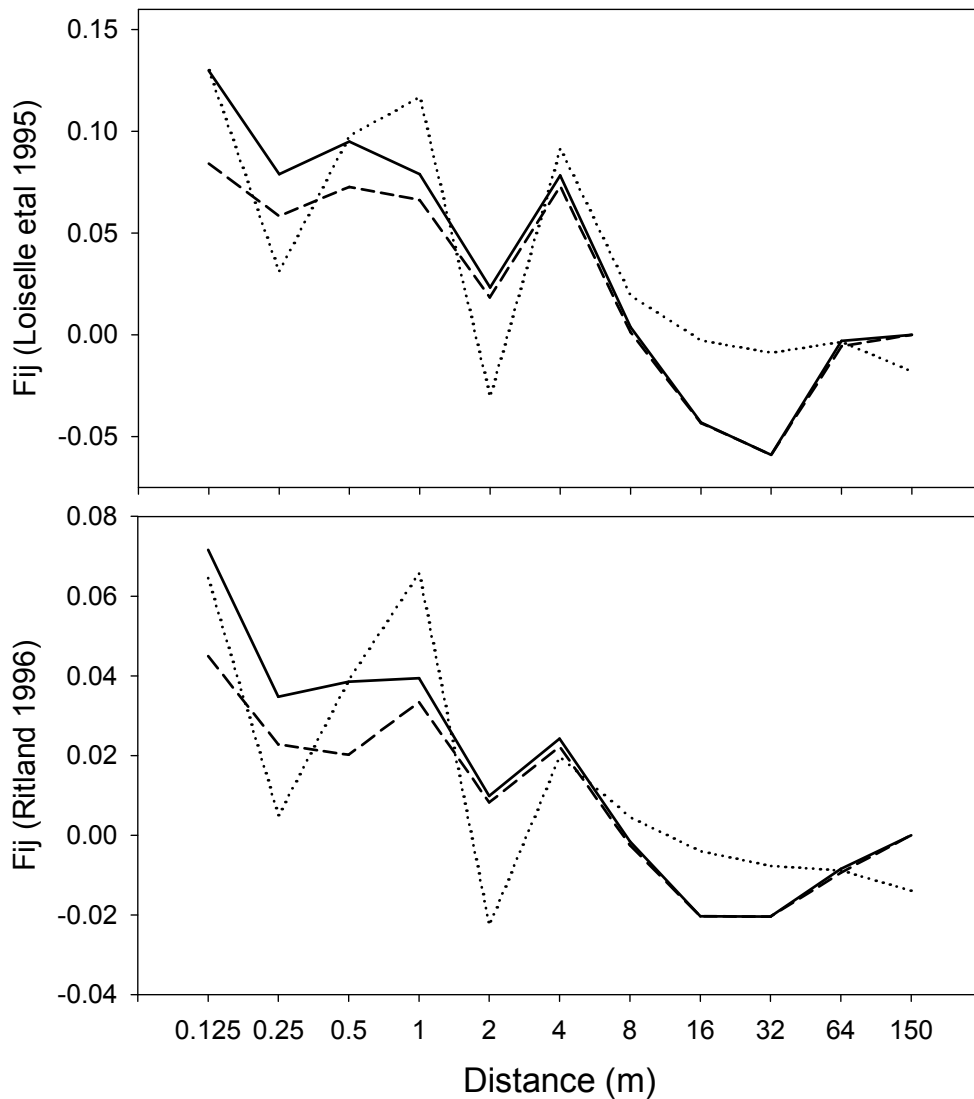
	Cd15		3	2.020	0.857	0.300	0.505	0.406
N	Cd4	18	2	1.906	0.668	0.556	0.475	-0.169
	Cd5		2	1.057	0.127	0.056	0.054	-0.029
	Cd7		5	2.541	1.208	0.667	0.606	-0.099
	Cd8		2	1.528	0.530	0.444	0.346	-0.286
	Cd15		3	2.323	0.918	0.611	0.569	-0.073
O	Cd4	15	2	2.000	0.693	0.733	0.500	-0.467
	Cd5		3	1.779	0.716	0.467	0.438	-0.066
	Cd7		3	1.411	0.563	0.200	0.291	0.313
	Cd8		2	1.301	0.393	0.267	0.231	-0.154
	Cd15		2	1.069	0.146	0.067	0.064	-0.034
All Pem	Cd4	21	2	1.849	0.652	0.333	0.459	0.274
	Cd5		2	1.265	0.365	0.143	0.210	0.319
	Cd7		5	1.423	0.663	0.238	0.297	0.198
	Cd8		2	1.208	0.314	0.190	0.172	-0.105
	Cd15		4	1.280	0.491	0.143	0.219	0.347

795 **S4. Summary table of geographic distances** between individuals grouped by the
 distance classes used in the spatial autocorrelation analysis (Figures 1 and 2 of main
 paper and S5 and S6 below). Double-letter codes represent the overhang to which
 both individuals belonged. Emboldened letters are between-overhang comparisons,
 where asterisks show the AMOVA derived pairwise p values from Table 3 of the
 800 main paper (p values represented by asterisks = * 0.050 – 0.011, ** 0.010 – 0.006,
 *** 0.005 – 0.001.).

0- 0.125m	0.126- 0.25m	0.26- 0.5m	0.51- 1m	1-2m	2-4m	4-8m	8-16m	16-32m	32-64m	64- 150m
GG	GG	GG								
KK	KK	KK	KK							
MM	MM	MM	MM							
HH	HH	HH	HH	HH						
II	II	II	II	II						
LL	LL	LL	LL	LL						
OO	OO	OO	OO	OO						
NN	NN	NN	NN	NN	NN					
JJ	JJ	JJ	JJ	JJ	JJ					
				LM*	LM*					
						GH				
						HI				
							GI			
							JK***			
								KL***		
								KM***		
								LN***		
								MN***		
									GJ	
									GK*	
									HJ	
									HK***	
									IJ*	
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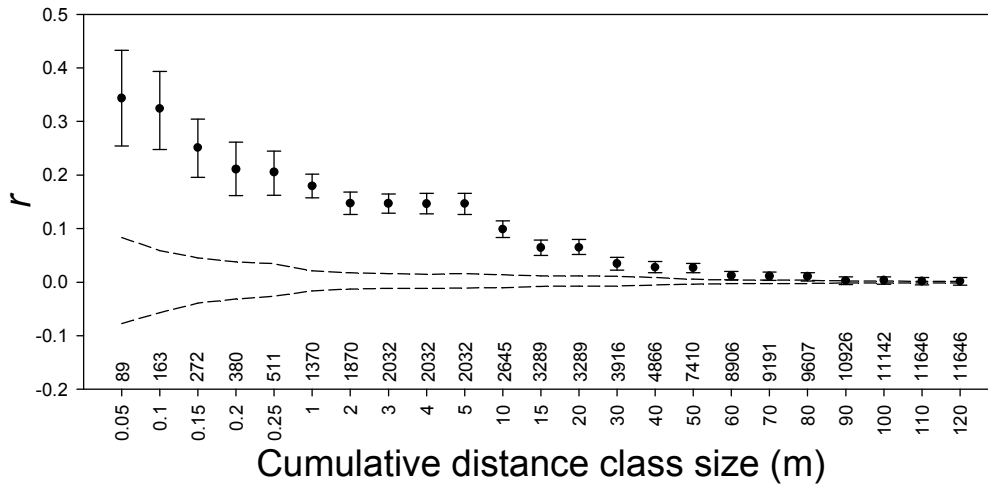
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805 **S5. Below figure.** Spatial autocorrelation analyses using the kinship coefficients
of Loisel et al. (1995 – upper figure) and Ritland (1996 – lower figure). Solid line
represents results for the ‘Full Wembury data set’ (= ‘ramet level’ analysis). Dashed
line shows results of the weighted approach to remove the distances among pairs of
identical genotypes from the data set. Where these two lines merge provides an
estimation of the clonal subrange. Dotted lines use central coordinates for each
810 replicated multilocus genotype.



S6. Below figure: The effect of increasing cumulative size class units on the interpretation of genetic structuring in the ‘full Wembury data set’. Confidence

815 intervals and error bars are as described for Figure 1. Note non-linear x-axis scale.



820 **S7. Mantel tests.** Mantel tests of matrix correspondence correlate the genetic and geographic distance matrices. Tests across the five data sets revealed the strongest significant positive relationship with the ‘full Wembury + Pembrokeshire data set’ ($r_{xy} = 0.137, p = 0.007$). Although weaker this was still significant when the Pembrokeshire outgroup was removed to leave the ‘full Wembury data set’ ($r_{xy} =$

825 $0.073, p = 0.021$). The 20 runs of the ‘random $n = 97$ ’ data set showed much variability in test results (mean $r_{xy} = 0.063, s.d. = 0.034$) but were dissimilar overall to the ‘one of each genotype’ data set (Mann-Whitney $W = 286.0, p = 0.014$: mean $r_{xy} = 0.040, s.d. = 0.008$). Only five of the ‘random $n = 97$ ’ and none of the ‘one of each genotype’ runs revealed a p value below the 5% threshold even when left uncorrected

830 for repeated testing. The ‘only unique genotypes’ data set showed the weakest relationship between the two variables ($r_{xy} = 0.023, p = 0.284$).

S8. A summary of the different hypotheses, tests and results of this study.

Topic	Hypotheses	Main statistical tests	Expected result if H ₀ is false	Actual result	Biological interpretation
Ryland's (1996) hypothesis that polyembryony would be less disadvantageous if mating neighbourhoods were genetically homogeneous	H ₀ : Genetic variation is partitioned randomly between and within overhangs H ₁ : Populations of <i>C. denticulata</i> are genetically <i>homogeneous</i> on the small geographic scales across which sperm would be routinely transferred (supports Ryland's hypothesis). H ₂ : Populations of <i>C. denticulata</i> are genetically <i>heterogeneous</i> on the small geographic scales across which sperm would be routinely transferred (does not support Ryland's hypothesis).	AMOVA	H ₁ : Little genetic variation would be apportioned to individuals within rock overhangs (= populations in the analysis). H ₂ : Significant amounts of genetic variation would be apportioned to individuals within rock overhangs (= populations in the analysis).	H ₂ = yes. A significantly high proportion of the genetic variation is apportioned to individuals within rock overhangs	Support for H ₂ . No support for H ₁ (Ryland's hypothesis). Thus <i>C. denticulata</i> is genetically heterogeneous over small spatial scales so colonies should be able to receive sperm from genetically diverse potential mates.
Within single rock overhang (<4m) genetic population structure	H ₀ : No population structure exists on this scale H ₁ : Isolation by distance exists	Spatial autocorrelation	Negative slope to correlograms at scales <4m.	Yes, negative slope to correlograms at scales <4m. Highest genetic similarity found at smallest spatial scale of ≤ 5cm.	Genetic structure can be detected within overhangs such that spatially close individuals tend to be genetically more similar.
Between different rock overhangs (4-133m) genetic population structure	H ₀ : No population structure exists on this scale H ₁ : Isolation by distance exists such that rock overhangs are genetically distinct from each other.	Spatial autocorrelation	Pairwise genetic similarity values are positive at within overhangs scales (<4m) but negative at scales that represent between overhang pairwise comparisons (>8m).	Yes, pairwise genetic similarity values are positive at within overhangs scales (<4m) but negative at scales that represent between overhang pairwise comparisons (>8m).	Individuals located on the same rock overhang tend to be genetically similar. Individuals located on different rock overhangs tend to be genetically distinct.
		AMOVA	Pairwise PhiPT values are significantly different from zero.	Yes, most (39/47) pairwise PhiPT values do show significant differentiation. Non-significant values may be an artefact of small sample size in some comparisons.	
Influence of polyembryony on population structure	H ₀ : Cloning cannot be detected. H ₁ : Cloning can be detected.	PI & PI _{sibs}	Number of individuals with shared multilocus genotypes > PI and PI _{sibs} estimates.	Yes, number of individuals with shared multilocus genotypes (= 90) > PI (1.4) and PI _{sibs} (19.5) estimates.	Cloning can be detected but seems to do little to increase the magnitude of pairwise genetic similarity at most within-overhang spatial scales except at distances between individuals of 1-2m, which may represent a common distance of cloned larval dispersal. Despite this the presence of repeat genotypes does lower the proportion of genetic variance that is allocated to individuals within overhangs, presumably because individuals with shared genotypes are likely to be found on the same overhang.
	H ₀ : Cloning makes no difference to population structure. H ₁ : Cloning does make a difference to population structure.	Spatial autocorrelation on the various Wembury datasets	The 'one of each genotype' dataset should produce correlograms that fall outside the 95% CI of the 'Full Wembury data set'. This pattern should not be evident with the 'random n=97' data set to exclude the effect of reduced sample size.	Mostly no, the magnitude of pairwise genetic similarity at most spatial scales remained similar in all three datasets, except at distances between individuals of 1-2m where positive genetic similarity was strongest when shared genotypes were included.	
		AMOVA on the various Wembury datasets	PhiPT measures should differ between the various Wembury datasets.	Probably yes, Phi PT values were highest when shared genotypes were included in the dataset, apportioning less of the genetic variation to individuals within rock overhangs.	

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