Living upside down: patterns of red coral settlement in a cave (#21242)

First revision

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Living upside down: patterns of red coral settlement in a cave

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Background. Larval settlement and intra-specific interactions during the recruitment phase are crucial in determining the distribution and density of sessile marine populations. Marine caves are confined and stable habitats. As such, they provide natural laboratory to study the settlement and recruitment processes in sessile invertebrates, including the valuable Mediterranean red coral *Corallium rubrum*. In the present study the spatial and temporal variability of red coral settlers in an underwater cave was investigated by demographic and genetic approaches. **Methods.** Sixteen PVC tiles were positioned on the walls and ceiling of the Colombara Cave, Ligurian Sea, and recovered after twenty months. A total of 372 individuals of red coral belonging to two different reproductive events were recorded. Basal diameter, height, and number of polyps were measured, and 7 microsatellites loci were used to evaluate the genetic relationship among individuals and the genetic structure. **Results.** Significant differences in the colonization rate were observed both between the two temporal cohorts and between ceiling and walls. No genetic structuring was observed between cohorts. Overall, high levels of relatedness among individuals were found. **Conclusion.** The results show that *C. rubrum* individuals on tiles are highly related at very small spatial scales, suggesting that nearby recruits are likely to by sibs and most larvae originated from adult colonies surrounding the tiles.

LIVING UPSIDE DOWN: PATTERNS OF RED CORAL SETTLEMENT IN A CAVE

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Abstract

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Methods. Sixteen PVC tiles were positioned on the walls and ceiling of the Colombara Cave, Ligurian Sea, and recovered after twenty months. A total of 372 individuals of red coral belonging to two different reproductive events were recorded. Basal diameter, height, and number of polyps were measured, and 7 microsatellites loci were used to evaluate the genetic relationship among individuals and the genetic structure.

Results. Significant differences in the colonization rate were observed both between the two temporal cohorts and between ceiling and walls. No genetic structuring was observed between cohorts. Overall, high levels of relatedness among individuals were found.

Conclusion. The results show that *C. rubrum* individuals on tiles are highly related at very small spatial scales, suggesting that nearby recruits are likely to by sibs and most larvae originated from adult colonies surrounding the tiles.

Introduction

Recovery and resilience of sessile benthic organisms mostly depend on their early life history stages such as dispersal, settlement and recruitment (Hughes et al., 2000; Pineda et al., 2007). It has been shown that abiotic (Torrents & Garrabou, 2011) and biotic (Lindsay, Wethey & Woodin, 1997) factors, as well as larval behaviour (Martínez-Quintana et al., 2014) may influence dispersal and larval mortality during the pre-settlement period. The settlement is influenced by events occurring during the planktonic stage (Babcock & Mundy, 1996). After the settlement, other sources of mortality (e.g. intraspecific competition, predation, detachment from the substrate) can affect the recruitment process (Perkol-Finkel et al., 2008; Santangelo et al., 2012). Larval dispersal and recruitment play a primary role in maintaining genetic diversity leading to a chaotic genetic patchiness (Johnson & Black, 1982). These effects are more evident at fine spatial scales below the expected range of larval dispersal of the species (Eldon et al., 2016). In marine invertebrates chaotic genetic patchiness seems related mainly to high variance in reproductive success (Hedgecock, 1994), collective dispersal (Broquet & Yearsley, 2012) and asynchronous local population dynamics (Eldon et al., 2016). Nevertheless, recruitment coming only or mostly from local populations (self-recruitment) can, lead to an impoverishment of the genetic variability and thus decreasing the population resilience to stressors (Brazeau, Sammarco & Atchison, 2011; Lasker, 2013) but could also enhance population survival through local adaptation (Sanford & Kelly, 2011).

Early life stages (from larval release to recruitment) in marine invertebrates such as sponges, ascidians and cnidarians, have been investigated using different tools such as laboratory experiments on larval behaviour (Guizien et al., 2012; Martínez-Quintana et al., 2014), on settlement and metamorphosis on different substrates (Bavestrello et al., 2000), field experiments

on settlement and post-settlement processes (Fraschetti et al., 2002), mathematical simulation by biophysical circulation modelling (Guizien et al., 2006), and empirical evidences from population genetics (Hedgecock, Barber & Edmands, 2007; Eldon et al., 2016). Moreover, recruitment rates, and their variability in space and time, can be estimated directly, using settlement tiles (Bramanti et al., 2007; Green & Edmunds, 2011; Santangelo et al., 2012; Bramanti & Edmunds, 2016); while spatial genetic structure (SGS; e.g. genetic variability, relatedness) can provide indirect estimates (Brazeau, Sammarco & Atchison, 2011; Smilansky & Lasker, 2014). The strong SGS observed in corals suggest that recruitment is often local, probably as a result of the short effective dispersal of larvae and their philopatric behaviour (Costantini, Fauvelot & Abbiati, 2007a; Ledoux et al., 2010a). However, to date, only few studies have analysed the SGS in coral settlers and recruits (Brazeau, Sammarco & Atchison, 2011; Torda et al., 2013; Smilansky & Lasker, 2014).

Underwater caves (sensu Rastorgueff et al., 2015) represent a naturally fragmented and confined habitat not exposed to the dominant currents, which provide a natural protection from disturbances associated with waterborne substances (Garrabou & Harmelin, 2002). Due to their high species richness, they are considered a Mediterranean biodiversity reservoir (Gerovasileiou & Voultsiadou, 2012). Underwater caves represent, therefore, an excellent natural mesocosm to investigate the recruitment processes without adding other stochastic external disturbances. Moreover, the understanding of recruitment processes in caves will be pivotal to forecast their ability to recover after disturbances, and to understand if they can act as refugia for species living outside the caves.

The red coral (*Corallium rubrum* L. 1758) is one of the abundant species inhabiting Mediterranean caves due to its preference for dim-light conditions and down-facing surfaces

(Laborel & Vacelet, 1961; Garrabou & Harmelin, 2002; Virgilio, Airoldi & Abbiati, 2006). Red coral is a gonocohoric species with internal fertilization. Gonadal development follows an annual cycle with a synchronized release in summer (Santangelo et al., 2003). Planulae are internally brooded and released once a year over a period of approximately 2 weeks between the end of July and early August (Santangelo et al., 2003; Bramanti et al., 2005). Natural (e.g., smothering by sediments, infection by parasites), anthropogenic stressors (e.g., harvesting: Tsounis et al., 2013, habitat loss and fragmentation) and threats arising from climate change (e.g., acidification: Bramanti et al., 2013; Cerrano et al., 2013; increasing of sea water temperature: Cerrano, Bavestrello & Bianchi, 2000) affect *C. rubrum* populations almost along its entire geographical distribution. Several recruitment studies using settlement tiles have been carried out on shallow-water red coral populations inhabiting vertical cliffs or small crevices (Bramanti, Magagnini & Santangelo, 2003; Bramanti et al., 2007; Santangelo et al., 2012). High variability in the density of recruitment between different sites has been found, and was attributed to biological interactions (e.g. competition for space, predation, overgrowth). Population genetic studies, conducted only on adult colonies, have shown large heterozygosity deficiencies, suggesting the occurrence of inbreeding (non-random mating) within populations (Costantini, Fauvelot & Abbiati, 2007a,b, Ledoux et al., 2010b,a; Aurelle et al., 2011) or demographic instability (Padron & Guizien, 2015) and a strong genetic structure at distances of less than one meter (Costantini, Fauvelot & Abbiati, 2007a; Ledoux et al., 2010a).

In the present study we investigated 2 cohorts of red coral recruits on settlement tiles deployed in a Mediterranean submarine cave acting as an experimental mesocosm. Specifically, we have analysed the variability of biometric parameters of the two cohorts of settlers (e.g. abundance of settlers, diameter, height, number of polyps). By means of microsatellite loci, the relatedness and

the fine spatial genetic structure within and between the two cohorts have been analysed in order to provide additional information on early life characteristics and population dynamics of this species. Understanding red coral recruitment processes is of key importance to unveil the drivers of the population dynamics of the species and hence the potential recovery of the overexploited/threatened populations.

Materials and methods

Study area and experimental design

The study was conducted inside the Colombara ~~(or Marcante)~~ cave (Lat 44° 18' 35", Long 9° 10' 37") located on the east coast of the Gulf of San Fruttuoso (Italy). The cave, 10 meters long, stands from 34 to 39 m depth on a rocky cliff southward oriented. The cave walls host a rich assemblage of sessile invertebrates typical of the sublittoral caves of the North-West Mediterranean Sea (Morri et al., 1986) with many sponges, corals, bryozoans, polychaetes, and tunicates.

Field experiments were approved by the Marine Protected Area of Portofino and by the Università Politecnica delle Marche (Authorization n. 3/2011 (n. prot. 449/2-1-5.) and authorization n° 4/2012 (Protoc. N° 409/2-1-1)).

In June 2010, about 3 weeks before the start of red coral spawning (Santangelo et al., 2003), sixteen 20 x 20 cm white PVC tiles, drilled in the centre, were fixed inside the cave by a steel screw. PVC was selected owing to the success in previous experiment on larval recruitment (Cerrano C, pers. comm; but see also Kennedy et al., 2017). Moreover, having positive buoyancy, the risk of detachment from the ceiling was avoided.

In order to test if recruitment is affected by orientation, one plot of four tiles was located on the right vertical wall, another plot on the left vertical wall, and two plots on the ceiling of the cave (Fig. 1). Each tile was attached to the rock, 1 m from the entrance and at a minimum distance of 30 cm from any another tiles, to avoid possible mutual interference. The red coral population distribution into the cave is patchy, showing an average density of 349 ± 215 col/m^2 (Cattaneo-Vietti, Bavestrello & Senes, 1993). Tiles were attached in low-density areas of the red coral population trying to limit as much as possible the breakage of surrounding colonies.

In February 2012, after two reproductive events (summer 2010 and summer 2011), the tiles were removed (n=14 as 2 located on one vertical wall were lost, Fig. 1). Recovered tiles were fixed in 80% ethanol and transferred to the laboratory. A picture of each tile was taken with a NIKON camera on a stereomicroscope NIKON SMZ 1500 and analysed with IMAGE J software version 1.24o (http://imagej.nihgov/ij) to study the spatial distribution of the red coral individuals. The position of each individual was marked and size (diameter) was measured by averaging the minimum and maximum width. All the individuals were then removed from the tiles and for each of them; the number of polyps was counted under the dissecting microscope. Polyps were removed from each individual and stored in plastic tubes with 80% ethanol at 4°C for the upcoming DNA extraction.

Based on the literature on red coral early life stages (Bramanti et al., 2005) the age of each individual was estimated on the basis of its height: individuals with an encrusting button shape and height equal to zero were assigned to the cohort 2011 (hereafter recruits); individuals that developed in height forming a small branch were assigned to the cohort 2010 (hereafter juveniles) (Fig. 2).

To check if a sort of cave-effect affected the pattern of recruitment, in the same period an additional series of plots with four PVC tiles were deployed on a vertical cliff out of the cave (Punta del Faro) where red coral population has the same range of density of the cave (425 ± 100 colonies x m^2) (Bavestrello et al., 2015). Plots were fixed at 70, 55, 45, and 35 m depth.

Measuring growth performances

The size structure of red coral individuals was obtained by analysing the frequency distributions of the basal diameter (for both recruits and juveniles) and of colony height (only for juveniles). The correlations between the parameters (diameter vs. height; diameter vs. number of polyps; height vs. number of polyps) were analysed by the Pearson's coefficient. Moreover, we tested the temporal and spatial variations of the abundance of individuals by two separate one-way ANOVAs with Cohort (two levels; fixed; recruits and juveniles) and Position (two levels; fixed; ceiling and walls) as factors using PRIMER v6 software program (Clarke & Gorley, 2006).

Measuring genetic variability and structuring

Due to the small number of *C. rubrum* individuals found on the tiles deployed on the walls, the molecular analysis was carried out only on the individuals occurring on the tiles deployed on the ceiling of the cave.

Total genomic DNA was extracted from each individual (1 to 4 polyps per colony) following the CTAB protocol and purified by standard chloroform procedure. Seven microsatellite loci COR9, COR46, L7, COR58, MIC20, MIC24, MIC26 (Costantini & Abbiati, 2006; Ledoux et al., 2010b) were amplified either as single locus using the protocol by Costantini & Abbiati, (2006) and by Ledoux et al., (2010b) or in multiplex using a QIAGEN Multiplex PCR Kit. Genotyping was

174 performed by MACROGEN INC. Allele sizing was determined with Peak Scanner v1.0
175 software. Genotypic linkage disequilibrium at each pair of loci for each was tested using FSTAT
176 v.2.9.3.2 (Goudet, 2001). Significance of each pairwise comparison was tested using 3360
177 permutations of the data.
178 Scoring errors due to stuttering, large allele dropout and null alleles were controlled with
179 MICROCHECKER version 2.2.3 (Van Oosterhout et al., 2004). Estimated frequencies of
180 putative null alleles were subsequently calculated for each locus using the expectation
181 maximization (EM) algorithm of Dempster, Laird & Rubin, (1977) implemented in FREENA
182 (Chapuis & Estoup, 2007). Red corals settlers sharing the same multilocus genotype (MLG)
183 were identified using GENALEX version 6.1 (Peakall & Smouse, 2006). Identical MLGs can
184 result from two distinct sexual reproduction events. To test this, the unbiased probability of
185 identity (P_{ID}) that two sampled individuals share identical MLG by chance (Kendall & Stewart,
186 1977) was computed. The total number of alleles (N_a), observed (H_o), and unbiased expected
187 (H_e) heterozygosities (Nei, 1973) were calculated for each locus and for each cohort of settlers
188 (either recruits and juveniles) using GENETIX software package version 4.03 (Belkhir et al.,
189 2000). Allelic richness was calculated after controlling for differences in sample size (N = 54),
190 using a rarefaction approach implemented in HP rare (Kalinowski, 2005). The *f* estimator of F_{IS}
191 (Weir & Cockerham, 1984) was computed, and significant departures from the Hardy–Weinberg
192 equilibrium were tested using Fisher's exact test in GENEPOP version 3.4
193 (http://genepop.curtin.edu.au/; Raymond & Rousset, 1995), with the level of significance
194 determined by a Markov chain randomization. Significant differences in genetic diversity (H_o, H_e
195 and F_{IS}) between recruits and juveniles were tested using a Student's t-test. For all the analyses,

196 when necessary, a correction for multiple tests was applied with a false discovery rate of 0.05
197 (Benjamini & Hochberg, 1995).
198 To determine whether individuals were more related than expected under panmixia, the R_{XY}
199 pairwise relatedness coefficient (Queller & Goodnight, 1989) was computed separately for
200 recruits and for juveniles. This index varies between 0 and 1 with R_{XY} = 1.0 for full-sib
201 relationships, R_{XY} = 0.5 for half-sibs and R_{XY}= 0 for unrelated individuals in an infinitely large
202 panmictic population (Peakall & Smouse, 2006). The observed mean and variance of R_{XY} were
203 compared with their expected distribution under the null hypothesis of panmixia using 1000
204 permutations of alleles as implemented in IDENTIX (Belkhir, Castric & Bonhomme, 2002). The
205 null distribution was obtained by a conventional Monte Carlo resampling procedure, which
206 randomly selected 10,000 genotypes without replacement and then recalculating the statistic.
207 Null hypothesis could be rejected with a significance level of 5%, given that the observed value
208 of the statistic was above the 95% level of the resampled statistics.
209 An exclusion test, performed in GENECLASS 2.0 (Piry et al., 2004) was used to test whether
210 individuals found in one tile was more similar to the individuals settled on the same tile. First,
211 the likelihood that an individual belongs to a particular tile was computed with a Bayesian
212 criterion of Rannala & Mountain (1997). Then, this likelihood was compared to a distribution of
213 likelihoods of 10000 genotypes simulated from each candidate tile with a Monte Carlo
214 algorithm. An individual was excluded from its tile when the probability of exclusion was
215 greater than 95% (P or $\alpha \leq 0.05$). The second part of this Bayesian analysis utilized the
216 probabilities that the individuals excluded from their sampling tile originated from one of the
217 other tiles. Thus, individuals that were excluded from their sampling tile when $P \leq 0.05$, were
218 assigned to another tile when $P \geq 0.1$.

219 The value of effective population size (*N*e) for each cohort was inferred using the standard
220 linkage disequilibrium method with Waples, (2006) correction. The computations were
221 performed with LDNe under the random-mating model, excluding rare alleles with frequencies
222 of less than 0.02 and using the Jackknife option to estimate confidence intervals (Waples & Do,
223 2008, 2010).
224 Genotypic differentiation between the recruits and juveniles was tested with an exact test
225 (Markov chain parameters: 1,000 dememorizations, followed by 1,000 batches of 1,000
226 iterations per batch), and the P value of the log-likelihood (G)-based exact test (Goudet et al.,
227 1996) was estimated in GENEPOP. The analysis was performed three times using three different
228 subsets of recruits, comparable to the number of juveniles, randomly selected.
229 A Bayesian approach implemented in the program STRUCTURE version 2.2 (Pritchard,
230 Matthew & Donnelly, 2000; Falush, Stephens & Pritchard, 2007) were used to estimate the
231 number of genetic clusters, K, within the entire sample (i.e. recruits + juveniles). Mean and
232 variance of log likelihoods of the number of clusters for $K = 1$ to $K = 10$ were inferred by
233 running structure ten times with 1000000 repetitions each (burn-in = 100000 iterations) under the
234 admixture ancestry model and the assumption of correlated allele frequencies among samples.
235 Due to the presence of null alleles, the clustering analysis was conducted on the original data set,
236 using the option of null alleles coded as recessive alleles described in Falush, Stephens &
237 Pritchard, (2007). Mean likelihoods of K from ten runs were plotted using STRUCTURE
238 HARVESTER 0.56.3 (available at http://taylor0.biology.ucla.edu/struct_harvest/). Results of all
239 runs were averaged in CLUMPAK server (Kopelman et al., 2015). Moreover, since the Structure
240 analysis could be inflated by the HW disequilibrium, a discriminant analysis of principal
241 components (DAPC), available in the Adegenet package (Jombart, Devillard & Balloux, 2010)

242 for R (R Development Core Team, 2012), was also performed. This technique is designed for
243 multivariate genetic data (multi-locus genotypes). It maximizes the variation between groups by
244 first performing a principal component analysis (PCA) on pre-defined groups or populations and
245 then uses the PCA factors as variables for a discriminant analysis (DA), thus ensuring their
246 independence.
247 Spatial autocorrelation analyses among individuals were performed with SPAGEDI (Hardy &
248 Vekemans, 2002). The Loiselle's kinship coefficient (ϕij; Loiselle et al., 1995) was used since it
249 is not dependent on Hardy–Weinberg (HW) equilibrium conditions (Hardy, 1999) and it has
250 been proved to be very effective in determining spatial genetic structure (Vekemans & Hardy,
251 2004). Two different analyses were performed. To test the spatial autocorrelation within the
252 cave, distance categories were set based on the distance between tiles and considering the
253 distance between two individuals found within the same tile as zero. Then, a spatial
254 autocorrelation analyses within those tiles containing more individuals (T4, T5, T7, T8, T9 and
255 T10; see results) were carried out. Taking into account the distance between individuals, the
256 distance categories were set in such a way that the number of pairwise comparisons within each
257 distance category was approximately constant. Statistical significance was based on permuting
258 individual locations among all individuals 10000 times and calculating upper and lower 95%
259 confidence interval for each distance class.
260
261 **Results**
262 *Red coral recruitment*
263 In the plots positioned into the cave, overall 372 individuals were observed on the 14 tiles.
264 *Corallium rubrum* settled on every tile deployed on the ceiling of the cave, but recruitment failed

on three out of eight tiles on the walls (T1, T13, T14). In fact, of the 372 individuals, 350 were found on the ceiling tiles, and only 22 on the tiles deployed on the walls, corresponding to a significantly higher density on the ceiling than on walls (ANOVA: $F_{1,14} = 10.78$; $P < 0.01$, Fig. 3). Of the 350 individuals on the ceiling, 278 were recruits and 72 were juveniles, corresponding to a density of 34.75 ± 23.86 /400 cm^2 and of 9 ± 6.82 / 400 cm², respectively (ANOVA: $F_{1,26} = 5.99$; $P < 0.05$, Fig. 2). The 22 individuals found in the wall were all recruits (Fig. 3). On the tiles positioned along the vertical cliff, at all depths no recruits were found when checking in summer 2011.

Growth performances

The size/recruit structure showed a monotonic and decreasing pattern, in which recruits in the first class (recruits with diameter <1.5 mm) represented the dominant class (Fig. 4A). Only three recruits (1%) had a diameter > 6 mm, and they might be formed by merging of two or more planulae (Fig. 2) as observed by Cerrano C, (pers. comm.). The number of polyps per recruit ranged from 1 to 22, with 70.66% of the recruits presenting 1-2 polyps, 22% between 3-4 polyps and 7.33% more than four polyps (mean ± SD = 2.32 ± 2.14 polyps/recruit). Pearson's coefficient showed a low correlation (r = 0.4) (Fig. 4C).

The size structure of juveniles showed a more variable trend, with diameter values ranging from 0.15 mm to 8.95 mm with an average value of 2.5 ± 1.5 mm (Fig. 5A). The number of polyps ranged from 0 to 22 (average number of 9.21 ± 4.6) (Fig. 5C). Juveniles with 3-4 polyps were the 9.7% of the total number, while 90.3% of them had more than five polyps. The height of the juveniles ranged from 0.5 to 9.15 mm, with a mean of 3.3 ± 1.8 mm (Fig. 5E). Pearson's correlation coefficients showed no correlation between diameter and number of polyps (r = 0.09,

Fig. 5B), nor between diameter and height (r = 0.03, Fig. 5F). A weak but significant correlation was observed between height and number of polyps (r = 0.3, Fig. 5D).

Genetic variability

Individuals in which more then two loci did not amplify do to technical failures (e.g. low DNA quantity, no amplification ~~of~~ after re-amplification)~~,~~ were not included in the dataset. A total of 290 red coral individuals were genotyped. No genotypic disequilibrium was observed between loci (all $P > 0.05$ after FDR correction). No evidence of scoring errors due to stuttering or large allele dropout was found, according to MICROCHECKER. An excess of homozygotes was detected at all loci due to the presence of null alleles. Null allele frequencies ranged from 0.09 (Mic24) to 0.25 (Cor9, Mic26 and Cor58). Number of alleles ranged from 10 (Mic20) to 23 (Cor9 and Mic26). The expected and observed heterozygosity ranged from 0.29 (Cor46) to 0.81(Cor9) and from 0.13 (Mic26) to 0.64 (Mic20), respectively. The estimators of F_{IS} (*f*) were positive and ranged from 0.21 (Mic20) to 0.86 (Cor48) (Table 1).

Overall, a low genetic variability (H_e= 0.59 ± 0.16 and H_o= 0.29±0.2; mean ± SD) was found. Out of the 290 individuals analysed, 281 different multilocus genotypes (MLGs) were identified. Four MLGs were found twice; one MLG was encountered three times and one four times. Individuals sharing the same MLG always came from the same tile. Tiles where identical MLGs were found were T7, T8 and T10. The individuals sharing the same MLG were between 0.2 cm and 13.65 cm away (in T7 and T10, respectively). In T8 and T10, one recruit and one juvenile shared a MLG. The unbiased probability of identity (P_{ID}) that two sampled individuals share identical MLG by chance was $1.5e^{-04}$.

No significant differences in genetic variability were observed between juveniles and recruits in terms of H_e and H_o (P values associated with the permutation procedure: P_{Ho}=0.65 and P_{He}=0.71); while recruits showed a significantly higher allelic richness than juveniles (P_{Ar}=0.001).

Relatedness

A high degree of genetic relatedness among individuals was found: a t-test performed on the mean observed relatedness, and test based on a permutation procedure (expected under panmixia) gave different responses (observed mean rxy=0.027, resampled mean rxy=0.014, t=1173, P = 0.001). Pairwise relatedness based on the Queller & Goodnight, (1989) coefficient revealed that 25.98% of the pairwise comparisons were involved in one or more parentage relationships, with 8.57% and 17.41% of individuals involved in a full-sib and half-sib relationship, respectively. These percentages were numerically similar considering the relatedness within the two temporal cohorts separately.

The percentage of individuals correctly assigned at the same tile where they were found by the individual assignments method using GENECLASS was around 50%. The effective population size (Ne) was estimated in 68.7 (95% CI: 42.5-116.4) including all the individuals and in 32.5 for recruits (95% CI: 20.6-57.8) and -1467.8 for juveniles (95% CI: 87.1 - ∞). The last negative value is expected when the population is sufficiently large that no notable linkage disequilibrium is induced through genetic drift (Waples & Do, 2010).

Spatial and temporal genetic structure

No genetic structuring was observed between recruits and juveniles using the three random dataset (F_{ST} = 0.008, P = 0.16). The clustering method identified K = 2 gene pools based on Evanno's delta K statistic (Supplemental Fig. S1, S2) but with high levels of admixture as many individuals could not be undoubtedly assigned to either cluster. Nevertheless, an eastward genetic gradient of differentiation were observed. The DAPC is in agreement with the Structure results (Fig. 6) with a higher genetic isolation of the titles T3 and T4 with all the other tiles.
Significantly positive kinship coefficients were detected between all the individuals within the single tile ($\phi ij = 0.066$, $p < 0.001$) indicating that individuals within tiles had a higher genetic relatedness than random pairs of individuals. Within the cave, the autocorrelogram suggested an estimated patch size of less than 40 cm (Fig. 7). The spatial autocorrelation between individuals within each tile showed a significant positive value within a range from 3 to 10 cm.

Discussion

In the present study the early life history traits of the Mediterranean red coral have been investigated using settlement tiles deployed inside a submarine cave. Size and genetic structures of recruits have been analysed. Two cohorts of red coral recruit were collected and they showed significant variability in space and time. No significant genetic structure was observed between the two cohorts, while settlers on the same tiles were highly related, suggesting that larval clouds recruiting nearby are sibs.

In summers 2010 and 2011 *C. rubrum* successfully recruited on artificial tiles inside the cave. The density of settlers (26.57±29,92 individuals/400 cm^2) was higher compare to values found by Bramanti et al., (2014) in Cap de Creus marine reserve (Costa Brava, Spain: 42°29.21' N; 03°30.18'E, Spain, 5.6±2.8 individuals/400 cm^2) and in Portofino (Eastern Ligurian Sea, Italy:

355 44°18.18’ N; 09°12.83’E, Italy. 17.5±4.7 individuals/400 cm^2). While adult colonies dwells on
356 the vault and on the walls of the cave, settlers density significantly differed between these two
357 habitats, with higher values on the tiles located on the ceiling of the cave. The only three tiles
358 (T1, T13, and T14) where no recruitment was recorded were positioned on the walls of the cave.
359 The causes of these differences are not fully understood yet, but they could be related to several
360 factors. Both larval behavior and habitat constraints can influence the higher recruitment density
361 observed on the ceiling. Even if *C. rubrum* larvae could survive at least 16 days (potentially up
362 to 42 days) in the plankton (Martínez-Quintana et al., 2014), inside caves and/or overhangs they
363 probably are trapped by the ceiling due to their negative geotaxis (Weinberg, 1979; Martínez-
364 Quintana et al., 2014). Sediment deposition is a limiting factor for red coral recruitment, and
365 observation made by Virgilio, Airoldi & Abbiati, (2006) showed that a thin coat of sediment
366 covering vertical surfaces, affects colony densities (but see also Cau et al., 2016). These findings
367 suggest that red coral populations dwelling in crevices and caves are more resilient due to
368 enhanced recruitment rates, while populations living on cliffs and on rocky bottoms, which are
369 the most exploited nowadays, might be endangered due to recruitment limitation.
370 Due to the difficulties in identifying tiny individuals (Bramanti, Magagnini & Santangelo, 2003),
371 only few studies have investigated the early life stage of this species (Garrabou & Harmelin,
372 2002; Bramanti et al., 2005, 2007). By using settlement tiles, it was possible to discriminate two
373 early life stages of *C. rubrum*: recruits and juveniles. Significant variation in the abundance of
374 recruits and juveniles were observed, suggesting both an inter-annual variability in larval supply
375 and/or post-settlement mortality. However, it is not easy to disentangle these two processes, and
376 this was not in the scope of this study. Concerning post-settlement processes, they include the
377 intra- and inter-specific interactions mediated by chemical cues, food limitation, local water

flow, predation and competition for space (Fraschetti et al., 2002). Competition for space, together with grazing intensity, is known to produce variations in coral recruitment at this spatial scale (Babcock & Mundy, 1996). All these processes, and their interactions, contribute to the high mortality rates in gorgonian recruits (Caley et al., 1996; Perkol-Finkel et al., 2008), including C. *rubrum* (Garrabou & Harmelin, 2002; Bramanti, Magagnini & Santangelo, 2003).

The number of polyps per individuals was consistent to Bramanti et al., (2005), with a higher number of polyps in juveniles compared to recruits, and a significant correlation between number of polyps and height in the juveniles. Moreover, individuals settling on tiles showed a considerable variability in diameter and height, suggesting that growth rate in early red coral stages may be extremely variable and possibly much higher than the growth rates estimated in adult colonies (0.89 mm /year between the first and second years, Bramanti et al. 2005; but see Table S2 in Cerrano et al., 2013). Considering that age of colonies on tiles is 20 months (for the juveniles) an average annual growth rate in diameter of 1.48 mmy^{-1} for juveniles was estimated. These results support the high variation in colony growth rates among geographic regions and in the early stages of colony life (Santangelo et al., 2012; Cerrano et al., 2013).

An important finding of the genetic characterization of settlers within the Colombara cave is that several identical multi locus genotypes (MLGs) were shared by recruits and juveniles. However, up to now, no evidence of asexual reproduction and/or polyp bail-out were reported in *Corallium rubrum*. The most likely explanation for the presence of identical MLGs is that, due to the low level of genetic variability of the species, these individuals are sibs sharing all the genotypes.

The low level of genetic variability observed compared to that previously observed in natural populations (Costantini, Fauvelot & Abbiati, 2007; Ledoux et al., 2010a), could be due to the small population size. However, this hypothesis seems unlikely considering the high density of

settlers on the tiles, and the high average density of colonies on the Portofino Promontory (227±37 colonies x m^2; Bavestrello et al., 2015), including the Colombara cave (Cerrano C. pers. obs.). The observed low genetic variability suggests high genetic drift acting within the cave. In fact, a correlation between low genetic variability and low effective population size was already observed in *Corallium rubrum* (Ledoux et al., 2015) confirming that the genetic drift might be relevant in this species (Costantini, Fauvelot & Abbiati, 2007; Ledoux et al., 2010a).

The significant deviations from Hardy–Weinberg equilibrium, emphasized by the high positive F_{IS} estimates it is unlikely to be related to null alleles. Null alleles were found in some loci but given their frequency, and previous observations in other red coral populations (Costantini, Fauvelot & Abbiati, 2007), their effect seem not to be so relevant. High inbreeding rates (e.g. mating between relatives) are a more likely explanation for the F_{IS} values. This phenomenon was already observed in *Corallium rubrum* (Costantini, Fauvelot & Abbiati, 2007) and was related to larval behaviour. This hypothesis is also supported by the occurrence of sibs on a single tiles, suggesting limited larval dispersal and/or collective dispersal (Broquet & Yearsley, 2012). Settlers' consanguinity could be explained by other factors (e.g., asynchrony of reproduction events, gametes behaviour, uneven sex ratio and clonality). Little is known about red coral gametes behaviour (Santangelo et al., 2003) but also the other possible explanations seem unlikely. In fact, in *Corallium rubrum* the sex ratio is balanced (Tsounis et al., 2006; Bramanti et al., 2014; Santangelo et al., 2015), reproduction is not completely synchrony but occurs within a discrete time-interval in summer (Santangelo et al., 2003) and up to now clonality (asexual reproduction) was not observed. Moreover, no genetic structure was observed between the two analysed cohorts of settlers. All these findings, including the high relatedness among settlers (full and half-sib relationship), suggest that in both cohorts larval supply was provided by a limited

number of genetically similar adult colonies (o progenitors). Fine spatial scale genetic structure is a common feature of gorgonians (Brazeau, Sammarco & Atchison, 2011), including red coral (Costantini, Fauvelot & Abbiati, 2007; Ledoux et al., 2010a). In the Colombara cave a population patch size of about 8 cm was detected, of the same range observed by Ledoux et al. (2010a) in a Mediterranean marine cave close to Marseilles (20-30 cm). These high SGS, together with the low genetic variability and the high inbreeding rate, in a close environment as a marine cave, could enhance local adaptation. No evidences of fitness variability due to inbreeding depression was observed in shallow red coral populations (Bramanti, Iannelli & Santangelo, 2009). However, further reduction of genetic diversity, and hence reduction of population size due to, for example, global changes (e.g. thermal anomalies, acidification), could lead to overcome the 'inbreeding threshold,' resulting in loss the of fitness and in a risk of local extinction (Frankham, 1995).

Conclusion

The present study provides new insight concerning recruitment processes in red coral populations using a submarine cave as an experimental mesocosm. The main outcome of the study is that *C. rubrum* individuals settling in the Colombara cave are highly related at very small spatial scales, and that most larvae recruiting nearby are sibs. Evidences of the processes explaining this pattern cannot be provided, however, self-recruitment and the presence of clouds of larvae that settle altogether could be possible explanations. Parentage analysis between adult individuals, both inside and outside the cave, and the recruits would help to disentangle the two processes. Understanding processes acting in the early life history of a species is a challenging but crucial task, with major implications for conservation. In fact, these processes drive the

447 population structure and dynamic of the species, and are essential for the resistance and
448 resilience of populations.
449
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Figure 1(on next page)

Maps of sampling location and scheme of the sampling design

A) Overview of the geographic location of the Colombara cave. B) zoom of the Portofino promontory where the cave is located. C) front of the cave and scheme of the tiles deployed. D) profile of the cave. White rectangles represent the tiles. Rectangles without number represent the lost tiles. Drawing made by Mancuso FP.

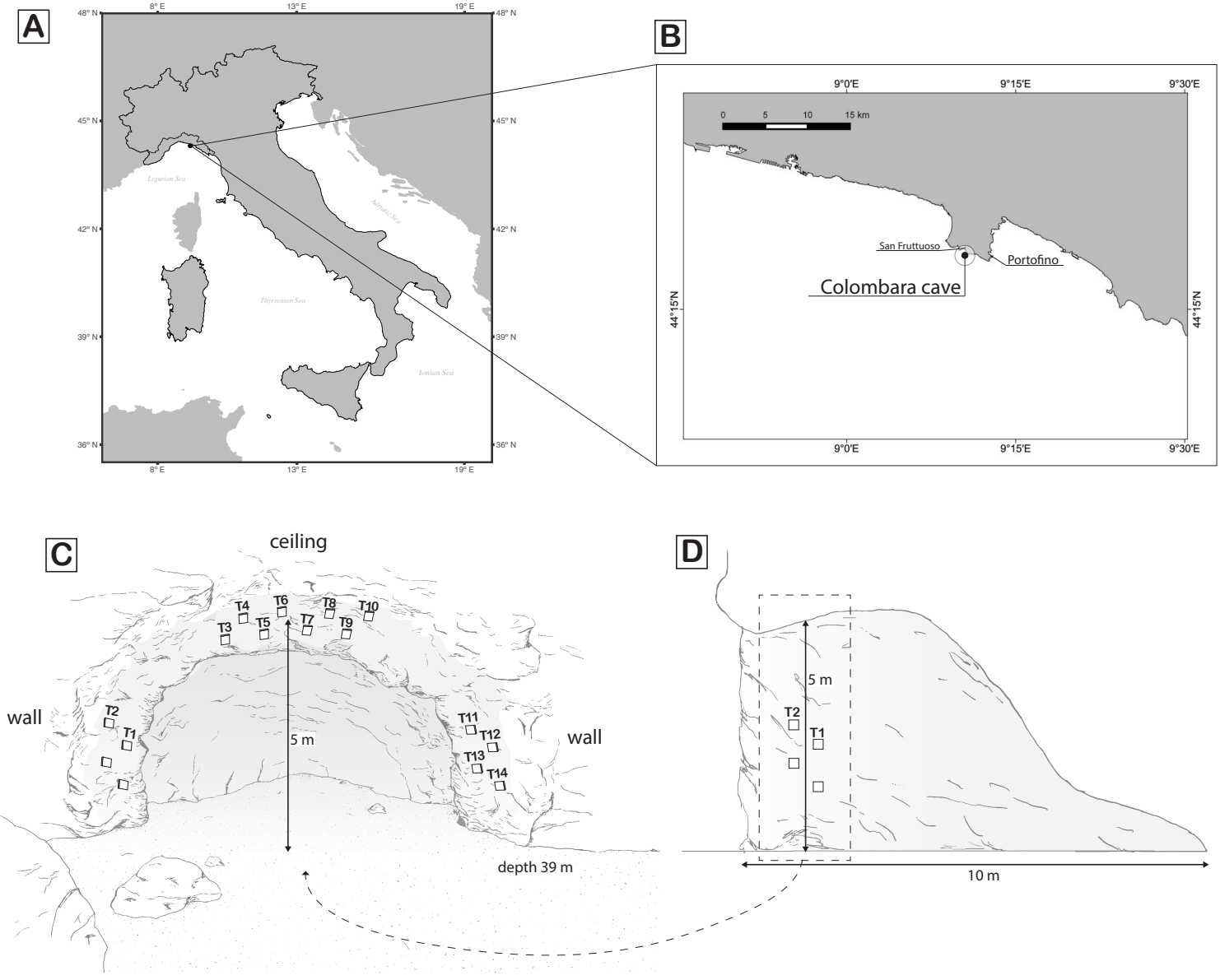

A
B
C
D
ceiling
wall
wall
depth 39 m
5 m
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
T11
T12
T13
T14
San Fruttuoso
Portofino
Colombara cave
10 m

Figure 2

Example of a PVC tile recovered from the Colombara cave after two years.

(A) Tile T7, (B) zoom of a recruit, (C) a juvenile, (D) a recruit probably derived by two merged planulae.

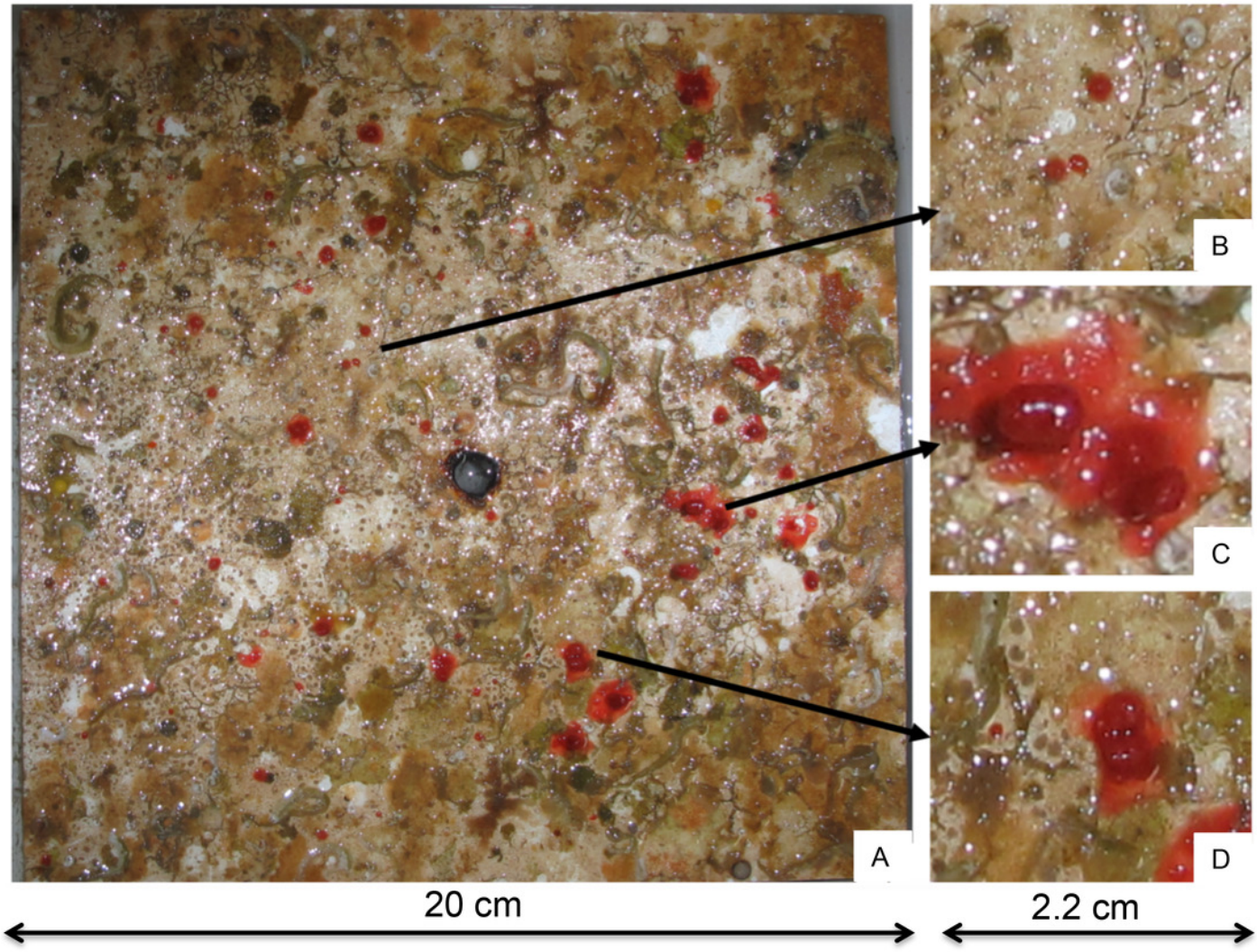


Figure 3(on next page)

Number of settlers on the 14 tiles deployed in the Colombara cave.

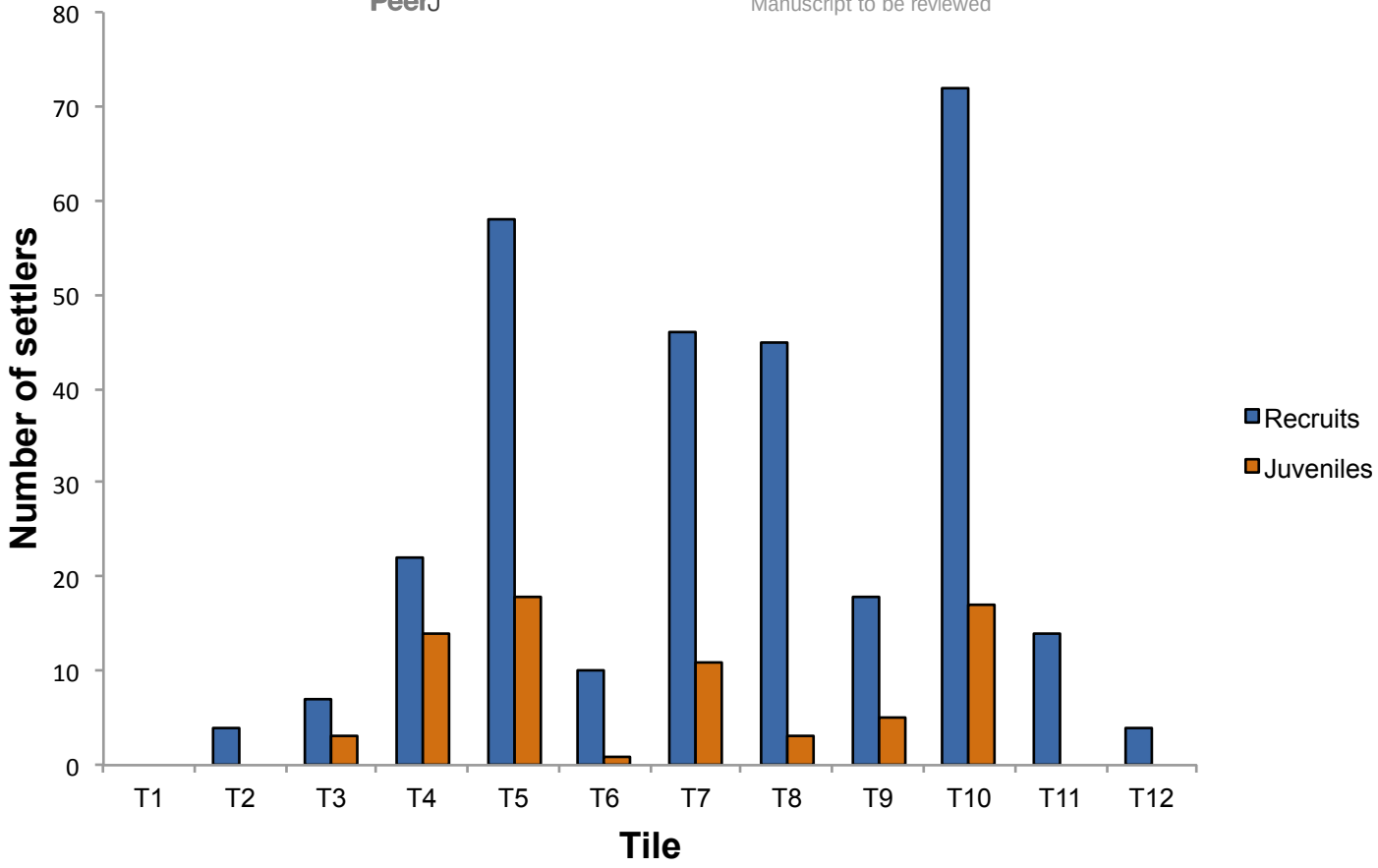
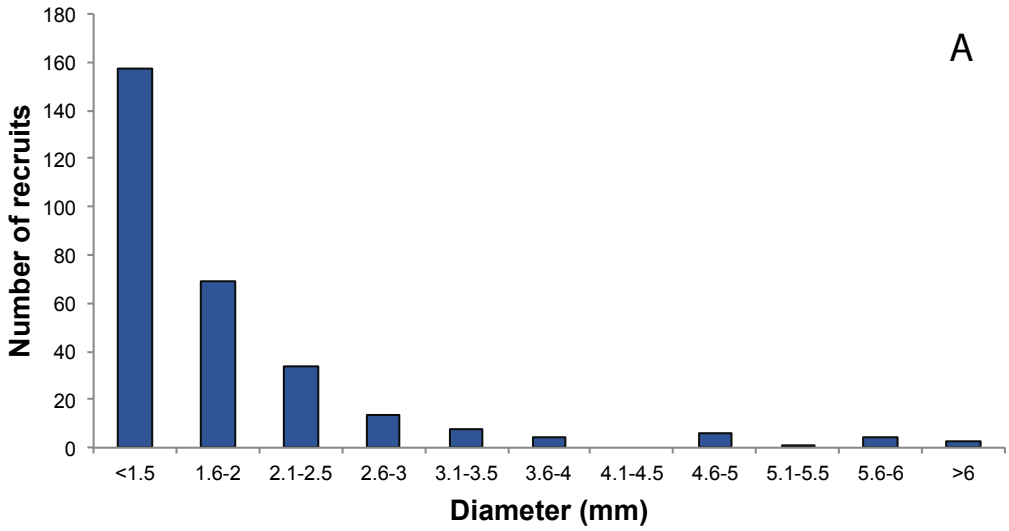
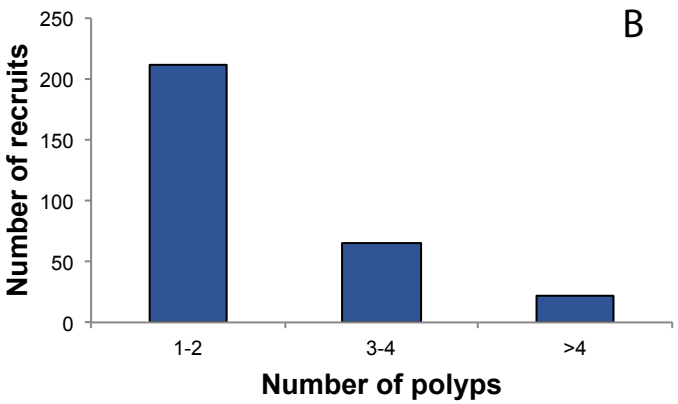

80
70
60
50
40
30
20
10
0
Number of settlers
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
T11
T12
Tile
Recruits
Juveniles

Figure 4(on next page)

Distribution of red coral recruits.

A) Diameter classes, B) number of polyp’s classes, C) relationship between number of polyps and diameter. r.: Pearson coefficient.


A
Number of recruits
180
160
140
120
100
80
60
40
20
0
<1.5
1.6-2
2.1-2.5
2.6-3
3.1-3.5
3.6-4
4.1-4.5
4.6-5
5.1-5.5
5.6-6
>6
Diameter (mm)


B
Number of recruits
250
200
150
100
50
0
1-2
3-4
>4
Number of polyps

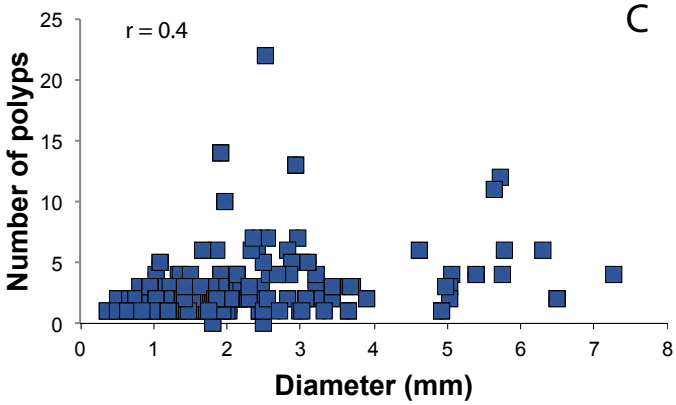
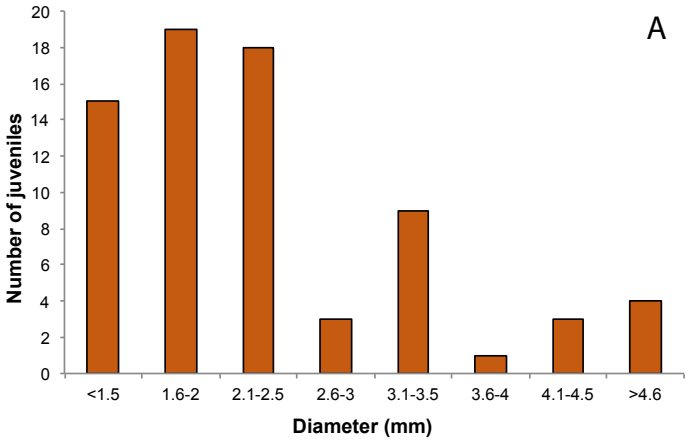
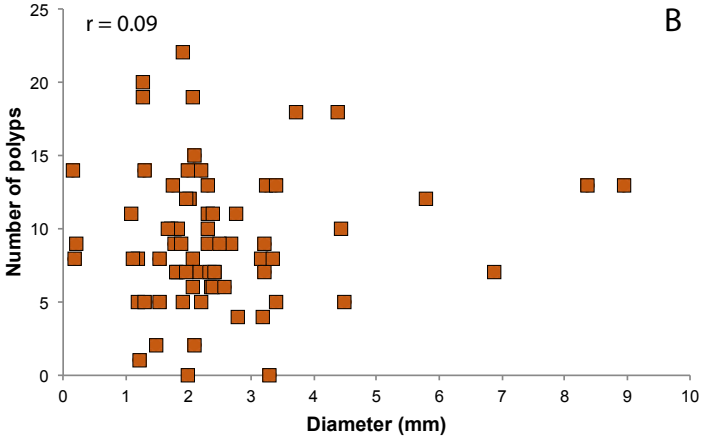

C
r = 0.4
Number of polyps
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
Diameter (mm)

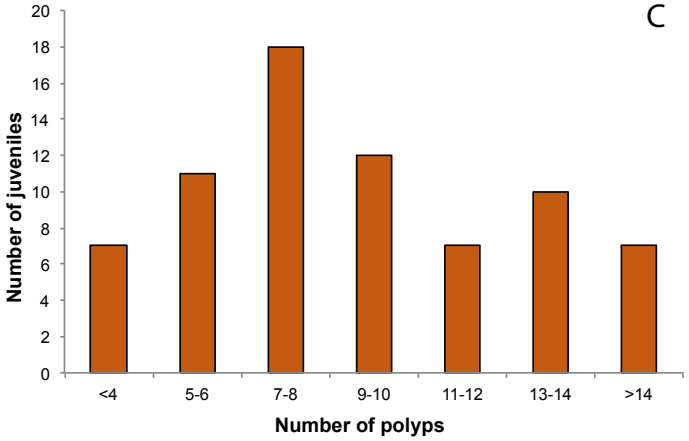
Figure 5(on next page)

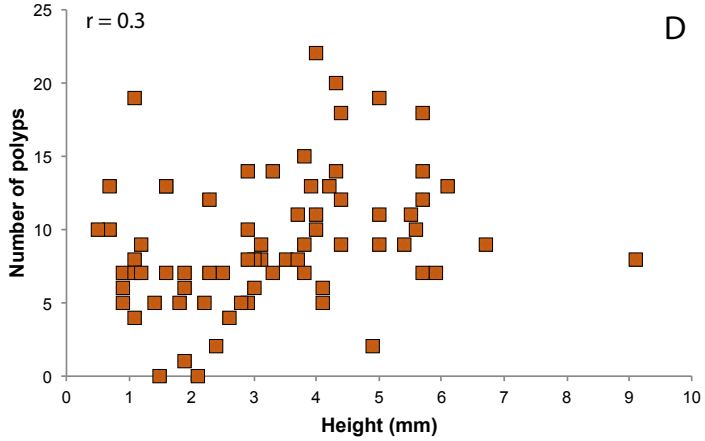
Distribution of red coral juveniles.

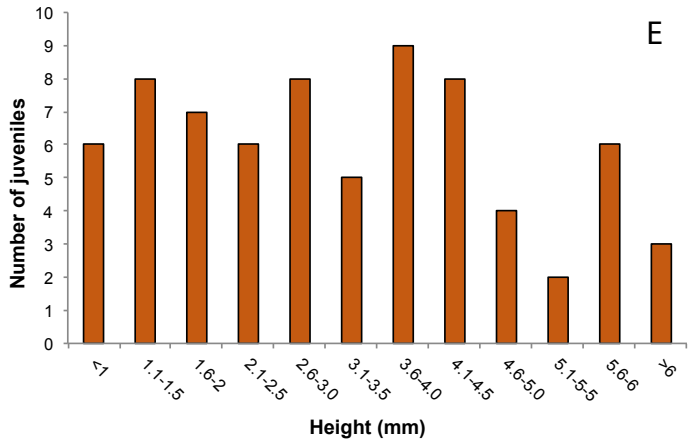
A) Diameter classes, C) number of polyps classes, E) height classes. Relationship between: B) number of polyps and diameter, D) number of polyps and height, F) height and diameter. r.: Pearson coefficient.


A
Number of juveniles
Diameter (mm)
<1.5
1.6-2
2.1-2.5
2.6-3
3.1-3.5
3.6-4
4.1-4.5
>4.6


B
r = 0.09
Number of polyps
Diameter (mm)


C
Number of juveniles
Number of polyps
<4
5-6
7-8
9-10
11-12
13-14
>14


D
r = 0.3
Number of polyps
Height (mm)


E
Number of juveniles
Height (mm)
<1
1.1-1.5
1.6-2
2.1-2.5
2.6-3.0
3.1-3.5
3.6-4.0
4.1-4.5
4.6-5.0
5.1-5.5
5.6-6
>6

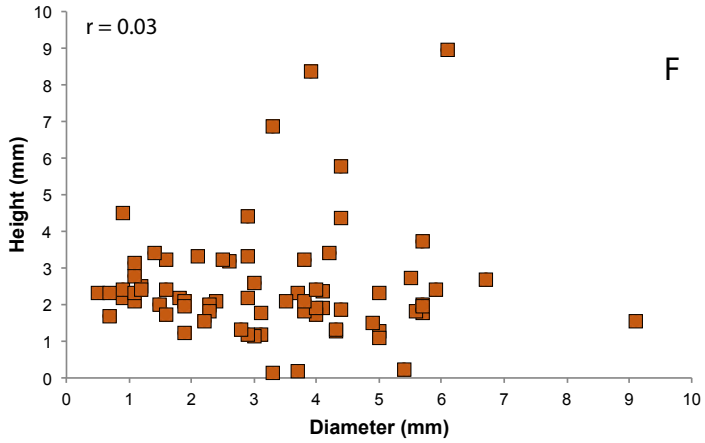

F
r = 0.03
Height (mm)
Diameter (mm)

Figure 6(on next page)

Scatterplot of the Discriminant analysis of principal components (DAPC) of the settlers found in the tiles deployed in ceiling of the Colombara cave.

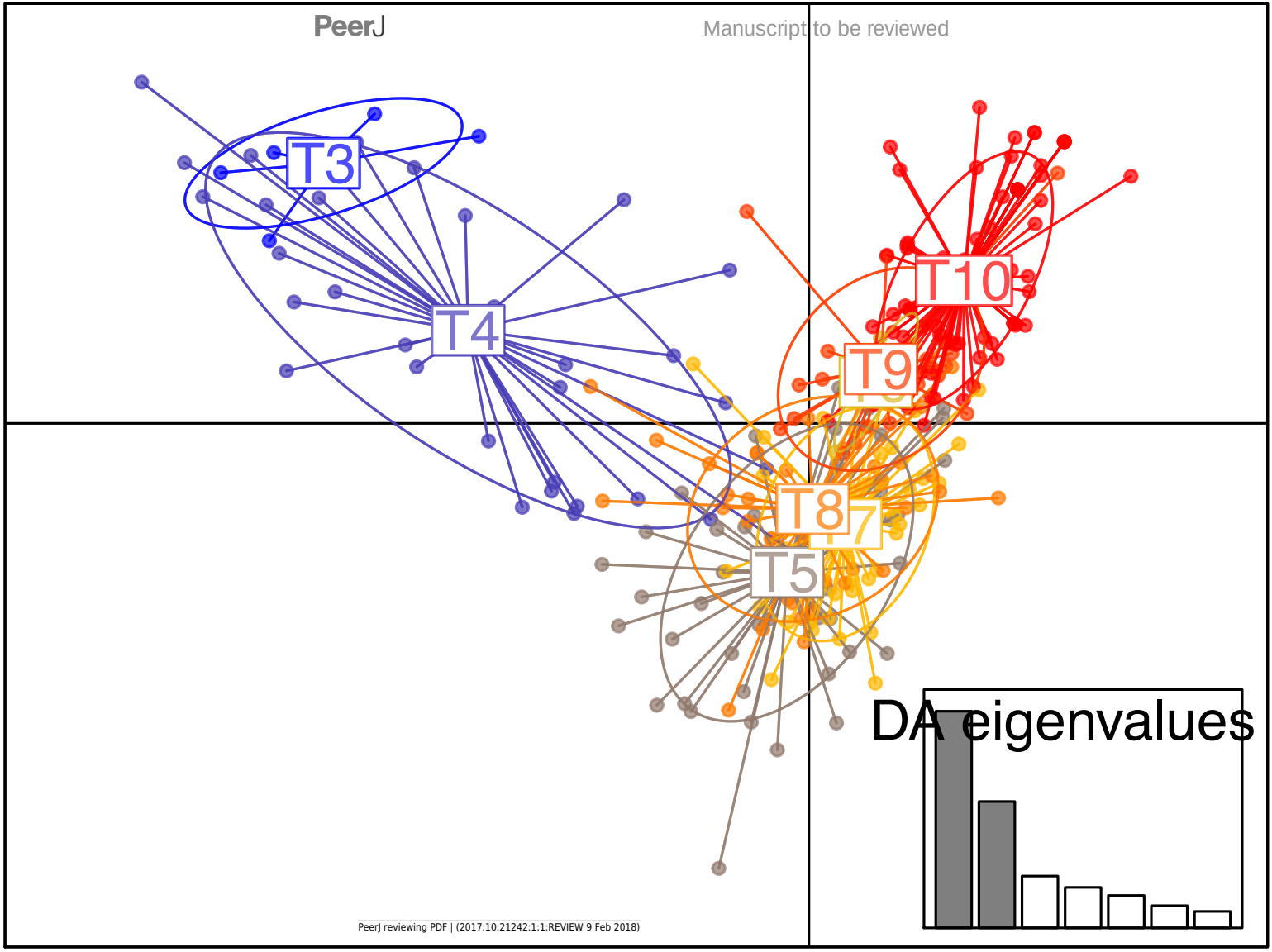

T3
T4
T10
T9
T8
T7
T5
DA eigenvalues

Figure 7 (on next page)

Spatial autocorrelation analysis of Loiselle kinship coefficient (Loiselle et al. 1995).

A) All tiles: correlogram performed for all the individuals within the cave B-G) correlogram performed for for each tiles with enough numbers of settlers (T4, T5, T7, T8, T9 and T10). Grey lines represent 95% confidence intervals.

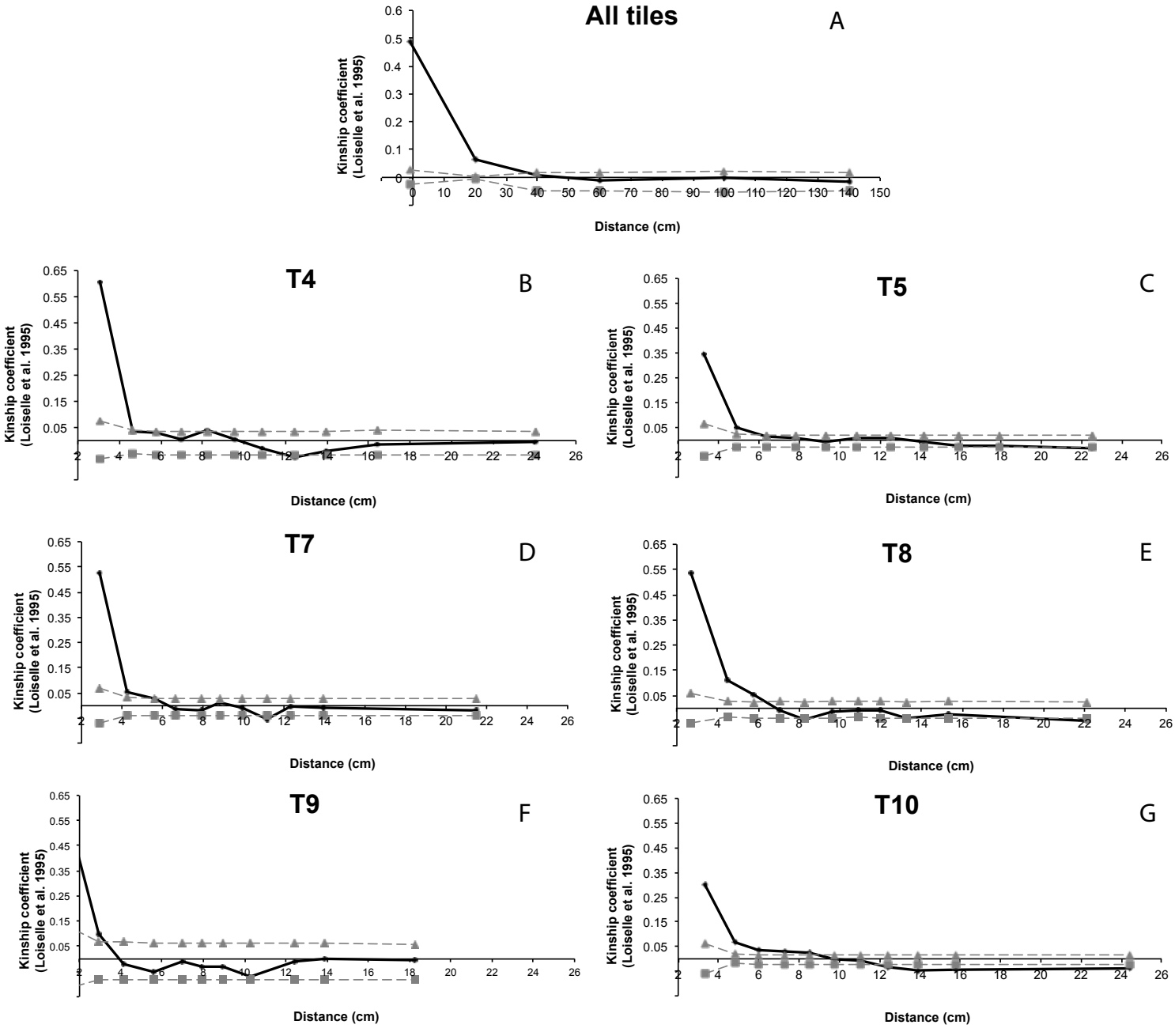

All tiles
A
Kinship coefficient (Loiselle et al. 1995)
Distance (cm)
T4
B
T5
C
T7
D
T8
E
T9
F
T10
G

Table 1(on next page)

Locus characteristics for all the individuals

Number of alleles per locus (Na); null allele frequency (r); gene diversity (H_e, Nei 1973); observed heterozygosity (H_o); Weir & Cockerham (1984) estimator of F_{IS} (*f*). * Significant deviation from panmixia after false discovery rate correction at 0.05 (Benjamini & Hochberg 1995).

Table 1: Locus characteristics for all the individuals: number of alleles per locus (Na); null allele frequency (r); gene diversity (H_e, Nei 1973); observed heterozygosity (H_o); Weir & Cockerham (1984) estimator of F_{IS} (*f*). * Significant deviation from panmixia after false discovery rate correction at 0.05 (Benjamini & Hochberg 1995).

	N	Na	R	H_e	H_o	*f*
Cor9	286	23	0.22	0.81	0.31	0.61*
Mic20	289	10	0.1	0.6	0.64	0.21*
Mic24	283	18	0.09	0.67	0.43	0.37*
Mic26	283	23	0.25	0.6	0.13	0.79*
Cor46	218	19	0.19	0.29	0.04	0.86*
L7	283	20	0.1	0.56	0.32	0.44*
Cor58	235	16	0.25	0.66	0.17	0.75*