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Inbreeding in the exploited limpet *Patella aspera* across the Macaronesia archipelagos (NE Atlantic): Implications for conservation

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ABSTRACT

The genetic erosion of populations exposed to human exploitation plays a detrimental role on a species ability to adapt to changing environmental conditions. The Macaronesia (NE Atlantic) endemic limpet *Patella aspera* (Röding 1798) has been subject to overexploitation throughout its geographic distribution. We analysed 841 limpet specimens from eleven islands across the archipelagos of Azores, Madeira and Canaries. Results from 11 nuclear microsatellite markers showed significant population structure between populations from Azores and populations from Madeira and Canaries, and absence of current or historic gene flow between these. M-ratios showed that both population clusters have experienced demographic changes over time. Heterozygote deficits were common across populations, which can be better accounted for by inbreeding than by null alleles or Wahlund effect. Such levels of inbreeding are likely a consequence of a significant reduction of reproductive units due to decades of intensive exploitation. As a sequential protandrous hermaphrodite, the size-selective harvesting of larger individuals likely fosters unbalanced sex-ratios and a consequent reproductive shortage. A recent compensatory hypothesis suggests that males are compensating the removal of larger females by undergoing sex change earlier and presumably at smaller sizes, as an adaptive response of the species under high size-biased fishing pressure. Despite such response, a dramatic reduction of *Ne* emerging from a large variation in the reproductive success due to overfishing and artificial genetic drift, can simply explain the inbreeding scenario observed in this Macaronesia endemic key species. This study provides valuable insights for management and conservation of *P. aspera* throughout Macaronesia.

1. Introduction

The most obvious consequences of living resources uptake by humans are the reduction of the targeted population size and biomass (*e.g.* Christensen et al., 2014). Fisheries research has long provided examples of such disruptive impacts on natural systems (Hutchings and Reynolds, 2004). At the population-level, fishing can alter size structure and population parameters in response to changes in species abundance (*e.g.* Jennings et al., 1999; Genner et al., 2010). Selective fisheries can also shift the community composition and the dynamics of an entire ecosystem, with known cascading effects on other taxa and biota (*e.g.* Mcclanahan et al., 1996; Myers et al., 2007; Casini et al., 2008; Smith et al., 2011). Moreover, the mean trophic level targeted by global fisheries has been decreasing over recent decades, shifting from large piscivorous fishes to smaller invertebrates and planktivorous fishes, leading to major changes in the structure of marine food webs (Pauly et al., 1998; Jackson et al., 2001).

Less noticeable are the genetic changes brought about by exploitation. These include the genetic subdivision of populations and the loss of genetic variation, which are thought to increase the risk of extinction via reductions in resilience and ability to recover following anthropogenic disturbances (Allendorf et al., 2008; Pinsky and Palumbi, 2014). In fact, fisheries-induced genetic changes are known to affect a number of life-history traits that often reduce the capacity for a

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population to recover. For example, the most usual size-selective fishing for larger individuals can negatively affect traits such as fecundity, maturation and larval growth in many marine organisms (Walsh et al., 2006; Swain et al., 2007).

As many other fisheries, the removal of limpets has a profound impact on the ecosystem. It is known that grazing by limpets not only determines macroalgal biomass overall (Hawkins et al., 1992), but also modifies ecosystem stability (Coleman et al., 2006). In fact, current dominance of intertidal algal turfs on many islands of Macaronesia is largely attributed to the virtual absence of patellid species due to overharvesting (Martins et al., 2008). Moreover, algal dominance means that there is little bare rock left for the settlement of new limpet recruits.

The most economically valuable limpet species across the region is *Patella aspera* (Röding 1798), which is present in all Macaronesia archipelagos with the exception of Cape Verde, and occurs on rocky shores from the lower intertidal down to 20 m depth. *P. aspera* is a protandrous (sequential hermaphrodite) species with external fertilization, that reaches sexual maturity around 40 mm in shell length (Martins et al., 1987). In such species, individuals start as males, with the majority switching later in life to female. *P. aspera* is more reproductively active during winter/early spring months with summer defined as a gonad maturation resting period (Martins et al., 1987; Vale 2016). Gametes are released into the water column and, upon fertilization, competent larvae can spend between to 2 to 32 days before they settle on hard substrate. These are temperature-dependent estimates that have been determined for other patellid species and may not reflect the true pelagic larvae duration of *P. aspera* (see Ribeiro, 2008). *P. aspera* is assumed to have speciated from its congeneric European continental form *Patella ulyssiponensis* (Koufopanou et al., 1999; Weber and Hawkins 2005) probably between 8 and 4 Ma (Sá-Pinto et al., 2008). Lack of gene flow between insular and continental populations, presumably after the establishment of extant ocean current patterns following the uplift of Panama Isthmus, has made *P. aspera* more vulnerable to exploitation.

A key challenge of fisheries management is the correct definition of effective stocks (Hawkins et al., 2016). This is vital to ensure that natural resources and the fishery are managed at the appropriate spatial scale. Although the reliability of such definition may be better achieved by using multiple stock identification techniques concurrently (*e.g.* see holistic approach in Begg and Waldman, 1999), molecular procedures have been more recently used to provide a genetic basis for aiding in stock identification (*e.g.* Griffiths et al., 2010; Dann et al., 2013). Moreover, molecular data can allow the understanding of the way populations in different geographic regions are connected via larval transfer or isolated from one another. In the event that connectivity exists among Macaronesia archipelagos, *P. aspera* could still maintain enough potential for local sustainability, provided that depleted stocks could be enhanced by natural gene flow from adjacent and/or more distant populations. However, experimental knowledge on such connectivity remains largely unknown so far. Here we used molecular data to identify patterns of genetic diversity and to test the null hypothesis of spatially homogeneous interbreeding and gene flow in *P. aspera* across Macaronesia, by studying the degree of genetic divergence between putative populations. We discuss possible implications of this study for fisheries management and conservation of such biologically, culturally and economically important endemic species across the region.

2. Methods

2.1. Sampling and laboratory procedures

A total of 841 individuals of *P. aspera* were collected across the Macaronesia archipelagos of Azores, Madeira and Canaries (Fig. 1). Individuals were labelled, and foot tissue samples were collected and preserved in 96% ethanol. Genomic DNA was extracted from muscle tissue using the E.Z.N.A. Mollusc DNA extraction kit following manufacturer's instructions (Omega Bio-tek). All DNA samples were quantified and checked for purity in a NanoDrop™ spectrophotometer (Thermo Scientific). Seventeen species-specific polymorphic microsatellite markers were amplified with fluorescently labelled primers following the PCR conditions described in Faria et al. (2015) (see Appendix A in the Supplementary material for methodological details).

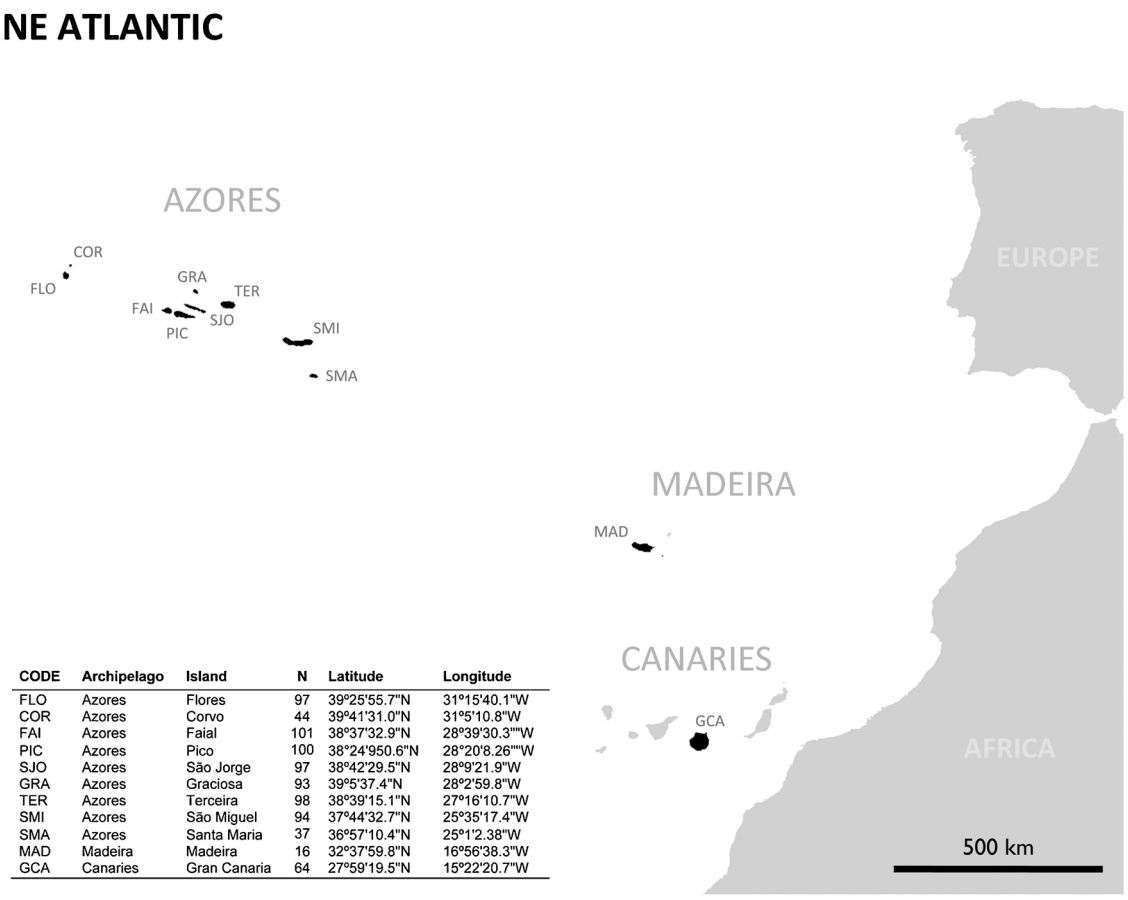
2.2. Genetic diversity

Standard genetic diversity measures such as allele frequencies, expected (H_E) and observed (H_O) heterozygosities and inbreeding coefficients (F_{IS}) within populations for each locus and over all loci were calculated using GENALEX v6.5 (Peakall and Smouse, 2012). Deviations from Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD) among all pairs of loci were tested using exact tests implemented in GENEPOP v4.1 (Raymond and Rousset, 1995). Because loci involved in significant linkage disequilibrium tests were detected, all subsequent analyses were performed on a reduced dataset of 11 loci. Allelic richness (A_R) and private allelic richness (A_P) were calculated in HP-Rare (Kalinowski, 2005) with a rarefaction sample size (in genes) of 10 due to missing data. Statistical significance for these parameters were tested with the Wilcoxon rank-sum test in R v3.3.0 (R Core Team, 2014). Results for multiple testing were adjusted by applying the false discovery rate (FDR) correction approach (Benjamini and Hochberg, 1995). As large heterozygote deficits are common in marine invertebrates (Addison and Hart, 2005), FREENA (Chapuis and Estoup, 2007) was used to detect the presence of null alleles and quantify their frequency. Evidence for null alleles can be found in deviations to HWE, more precisely in the significant excess frequency of homozygous genotypes. Neutrality of the markers was tested using LOSITAN software (Antao et al., 2008) (see Appendix A in the Supplementary material for methodological details).

2.3. Population structure

Genetic differentiation among population was estimated from the pairwise F_{ST} using the so-called ENA method described in Chapuis and Estoup (2007). This approach aims to correct for the positive bias induced by the presence of null alleles on F_{ST} estimation; 95% confidence intervals for the F_{ST} values were obtained using 10 000 bootstrap iterations and F_{ST} estimates obtained with and without applying the ENA algorithm were compared by means of a two tailed *t*-test. For comparative purposes, pairwise F_{ST} resulting from an analysis of molecular variance performed between each pair of populations (AMOVA) was also estimated in GENODIVE v2.0b25 (Meirmans and Van Tienderen, 2004); statistical significance was tested by means of a permutation procedure across loci, followed by the FDR correction for multiple testing. Genetic differentiation between populations was also determined using the D_{est} estimator (Jost, 2008) implemented in the R package DEMETICS v.0.8.4 (Gerlach et al., 2010) and P-values were estimated by bootstrap analysis (1 000 replicates). To test for isolation by distance (Wright, 1943), linearized $F_{ST\text{-}ENA}$ transformation ($F_{ST}/[1 - F_{ST}]$) was regressed onto the natural logarithm of geographic distance (GD) (Rousset, 1997) in R and tested for significance with a Mantel permutation procedure. POWSIM v.4.0 (Ryman and Palm, 2006) was used to determine the power of the markers to detect significant genetic differentiation at various levels of F_{ST} (see Appendix A in the Supplementary material for methodological details).

Genetic population structure was assessed using the Bayesian clustering algorithm implemented in STRUCTURE v2.3.4 (Pritchard et al., 2000) (see Appendix A in the Supplementary material for method details). The most likely number of K pools was selected using the ΔK method described in Evanno et al. (2005) and implemented in STRUCTURE HARVESTER v0.6.94 (Earl and vonHoldt, 2012). The results of ten replicate runs for each value of K (putative number of gene



CODE	Archipelago	Island	N	Latitude	Longitude
FLO	Azores	Flores	97	39°25'55.7"N	31°15'40.1"W
COR	Azores	Corvo	44	39°41'31.0"N	31°5'10.8"W
FAI	Azores	Faial	101	38°37'32.9"N	28°39'30.3""W
PIC	Azores	Pico	100	38°24'950.6"N	28°20'8.26""W
SJO	Azores	São Jorge	97	38°42'29.5"N	28°9'21.9"W
GRA	Azores	Graciosa	93	39°5'37.4"N	28°2'59.8"W
TER	Azores	Terceira	98	38°39'15.1"N	27°16'10.7"W
SMI	Azores	São Miguel	94	37°44'32.7"N	25°35'17.4"W
SMA	Azores	Santa Maria	37	36°57'10.4"N	25°1'2.38"W
MAD	Madeira	Madeira	16	32°37'59.8"N	16°56'38.3"W
GCA	Canaries	Gran Canaria	64	27°59'19.5"N	15°22'20.7"W

Fig. 1. Map of sampling locations for *Patella aspera* collected from the Macaronesia archipelagos of Azores, Madeira and Canaries (NE Atlantic).

pools) from 1 to 11 were averaged in CLUMPP v1.1.2 (Jakobsson and Rosenberg, 2007) and summary outputs were displayed graphically in DISTRUCT v1.1 (Rosenberg, 2004). Analyses were also conducted on a reduced dataset by excluding loci with a higher frequency of null alleles (*e.g.* Baums et al., 2012). Because the high frequency of null alleles at most loci and populations might lead to overestimation of the number of K pools, the robustness of STRUCTURE results was tested using another Bayesian inference method provided by GENELAND (Guillot et al., 2005) and implemented in R package (see Appendix A in the Supplementary material for methodological details). AMOVA as implemented in ARLEQUIN v3.5.2.2 (Excoffier and Lischer, 2010) was also used to characterize the genetic structure and variance within and between clusters identified in STRUCTURE and GENELAND analyses; significance was tested after 1000 permutations. Genetic diversity statistics were applied to each of the clusters identified with Bayesian analyses of population structure.

2.4. Unbiased estimates of inbreeding

Because homozygosity in *P. aspera* can be a consequence of harvesting-related inbreeding, the individual inbreeding model (IIM) implemented in the software INEST v2.0 (Chybicki and Burczyk, 2009) was used to simultaneously estimate null allele frequencies and inbreeding coefficients (fixation index, $F_{IS\text{-}INEST}$). This Bayesian approach allowed the calculation of unbiased estimates of inbreeding within a population after accounting for null alleles. Within INEST, two models were tested: firstly the full model which accounts for genotyping failures (*b*), inbreeding (*f*) and null alleles (*n*) and secondly the random mating model (*i.e.* when *f* is fixed at 0). The Deviation Information Criterion (DIC) is then used for model comparison. Support is given to an inbreeding effect when the lowest DIC is found in the *nfb* model. Also, to evaluate the effect of null alleles on F-statistics, uncorrected F_{IS} for each population was regressed onto the frequency of null alleles.

2.5. Bottleneck detection and gene flow

The heterozygosity excess test in BOTTLENECK v1.2.02 (Cornuet and Luikart, 1996) was used to test for evidence that populations had experienced recent genetic bottlenecks. Demographic declines were also assessed for the inferred clusters using the M-ratio test as implemented in *M_p_val* (Garza and Williamson, 2001). Recent migration among clusters was estimated using the Bayesian assignment test implemented in BAYESASS v3.0. (Wilson and Rannala, 2003). All bottleneck and migration analyses were repeated after discarding loci with > 20% proportion of null alleles (see Dakin and Avise, 2004) (see Appendix A in the Supplementary material for bottleneck and migration analyses details).

3. Results

3.1. Genetic diversity

All microsatellite loci were highly polymorphic with no significant differences in allelic richness among populations (single locus A_R ranging between 1.8 and 6.6; Tables B1 and B2). Conversely, private allelic richness (A_P) was significantly higher in the Canaries population ($P < 0.05$) in most pairwise comparisons. Observed heterozygosity was relatively low and similar across populations with a mean value of 0.309 ± 0.165 SD (Table B1). The expected mean heterozygosity was 0.691 ± 0.154 SD and all loci deviated from HWE. The estimated frequency of null alleles ranged from 0.07 to 0.36. There was no evidence for selection at any locus (Fig. B1).

Table 1
Pairwise estimates of $F_{ST\text{-}GENODIVE}$ (above diagonal) and Jost's D_{est} (below diagonal) among *Patella aspera* populations (see Fig. 1 for population codes). Significant values after FDR correction are in bold.

	COR	FLO	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA
COR	–	**0.011**	0.005	**0.010**	**0.009**	0.005	**0.010**	0.003	0.000	**0.026**	**0.086**
FLO	**0.042**	–	**0.009**	**0.005**	**0.005**	0.005	0.003	**0.008**	**0.008**	**0.060**	**0.114**
FAI	0.035	**0.034**	–	**0.006**	**0.007**	0.000	0.011	**0.006**	**0.013**	**0.035**	**0.104**
PIC	**0.050**	**0.028**	0.020	–	0.001	0.000	0.004	0.001	**0.011**	**0.042**	**0.104**
SJO	**0.053**	**0.031**	**0.035**	0.014	–	0.000	0.003	0.004	**0.010**	**0.033**	**0.092**
GRA	0.035	0.019	0.008	0.003	0.008	–	0.004	0.001	**0.008**	**0.031**	**0.097**
TER	**0.044**	**0.019**	**0.038**	0.025	0.021	0.020	–	0.003	0.006	**0.057**	**0.111**
SMI	0.022	**0.035**	0.021	0.015	**0.027**	0.013	**0.031**	–	0.002	**0.035**	**0.099**
SMA	0.011	0.043	**0.063**	**0.062**	**0.061**	**0.060**	**0.045**	0.031	–	**0.045**	**0.102**
MAD	**0.183**	**0.233**	**0.171**	**0.185**	**0.163**	**0.164**	**0.245**	**0.171**	**0.212**	–	0.020
GCA	**0.299**	**0.337**	**0.319**	**0.318**	**0.282**	**0.296**	**0.341**	**0.293**	**0.339**	0.104	–

3.2. Population structure

Significant genetic differentiation (after FDR correction) was found in 34 out of 55 pairwise $F_{ST\text{-}GENODIVE}$ comparisons (Table 1). $F_{ST\text{-}GENODIVE}$ ranged from 0 to 0.114 and was not significantly different from $F_{ST\text{-}FREENA}$ corrected for null alleles (*t*-test, $P > 0.05$). Higher values were mainly observed between Azores and Madeira plus Canaries. D_{est} estimates were proportionally higher than F_{ST} estimates and yielded similar statistical significance values (Table 1).

Regression analysis showed that *F*-statistics might have been highly overestimated in the presence of null alleles (Fig. B2) and can therefore affect F_{ST} estimates and their significance among populations, especially on those that are putatively more closely related such as the Azorean populations. Power analysis in POWSIN indicated that the number of individuals and the number of loci used in this study provide strong support to identify weak differentiation among populations at a true F_{ST} as low as 0.0025 with a statistical power of 100% for both Fisher's exact test and the chi-square test.

There was a strong signature of isolation by distance among archipelagos (Fig. 2, $r^2 = 0.541$; $P < 0.001$), but genetic differentiation within the Azores did not seem to relate to geographic distance (Fig. 2, $r^2 = 0.069$; $P > 0.12$). STRUCTURE analyses performed under mixed ancestry and sampling locations as prior information, and the ΔK metric of Evanno et al. (2005) revealed that $K = 2$ was the most likely number of gene pools/clusters for *P. aspera* in Macaronesia (Figs. 3 and B3, respectively). Two distinct clusters were identified: one involving all Azorean populations and another grouping Madeira and Canaries. Population structure became less evident when analytical parameters and settings were changed in STRUCTURE, especially when prior information for population origin was not implemented (see Fig. B4). The spatial model in GENELAND, bearing a correction for null alleles, estimated the number of clusters for *P. aspera* in Macaronesia as two, in agreement with initial STRUCTURE results (Fig. 3): a northern group encompassing all Azorean populations and a southern group including Madeira and Canaries populations. A significant genetic differentiation among clusters was also detected through AMOVA analyses; most of the total variance was due to variation within populations (Table B3).

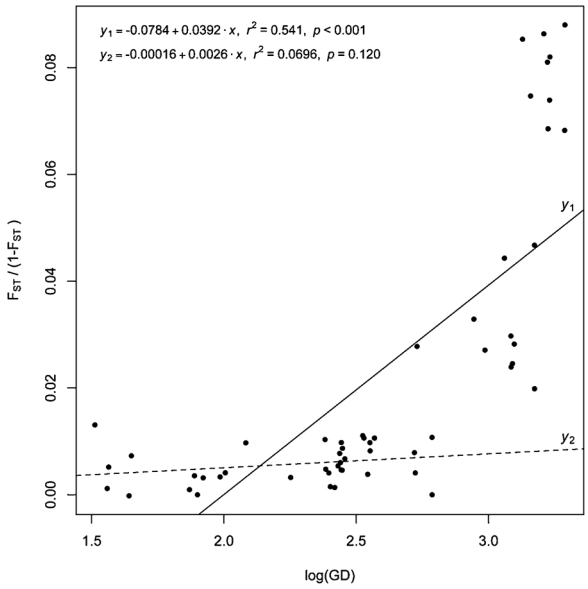


Fig. 2. Regression between genetic distances $F_{ST}/(1 - F_{ST})$ and geographical distances (GD) among and within archipelagos (y1 and y2, respectively).

The genetic diversity of each cluster was similar to those obtained for population/location level analyses (Table 2). Mean observed and expected heterozygosity in the first cluster (all samples from Azores) were 0.315 ± 0.160 SD and 0.701 ± 0.150 SD, respectively. For the second cluster (Madeira and Canaries samples), the observed mean heterozygosity was 0.285 ± 0.173 SD and the expected mean heterozygosity was 0.697 ± 0.188 SD.

3.3. Inbreeding

The multilocus "null free" average inbreeding coefficient (F_{IS}) as calculated with INEST varied between 0.113 and 0.497 (Table B1). Model comparison using INEST suggests that the heterozygote deficit observed in the dataset is better accounted for by inbreeding than by null alleles. In all cases, the *nfb* model had the lowest DIC (Table B4). As expected, regression analyses indicated a major effect of null alleles on uncorrected *F*-statistics (Fig. B2). Similarly, model comparison with INEST on the two clusters identified through Bayesian analyses also indicated that heterozygosities deficits are more related to inbreeding rather then to null alleles presence: the "null free" inbreeding coefficient $F_{IS\text{-}INEST}$ for each cluster was 0.399 (95% confidence interval: 0.368-0.431) and 0.104 (95% confidence interval: 0.043–0.272), respectively (Table 2).

3.4. Bottleneck detection and gene flow

The heterozygosity excess test implemented in BOTTLENECK did not find evidence for a recent demographic bottleneck in any of the clusters assumed (Table B5; $P > 0.05$). Bottleneck detection using the M-ratio method did, however, show that clusters have experienced demographic changes in time. Although the performance of the method is dependent on initial assumptions and settings, the more conservative approach recommended by Garza and Williamson (2001) indicated a recent reduction in population size, within the last few hundred generations, for both Azores and Madeira plus Canaries clusters (Fig. B5). In such case M-ratios for individual clusters fell under the lower 5% of the distribution of simulated M-values. Setting the average size of non one-step mutations to 2.8, which is the mean value for this parameter in

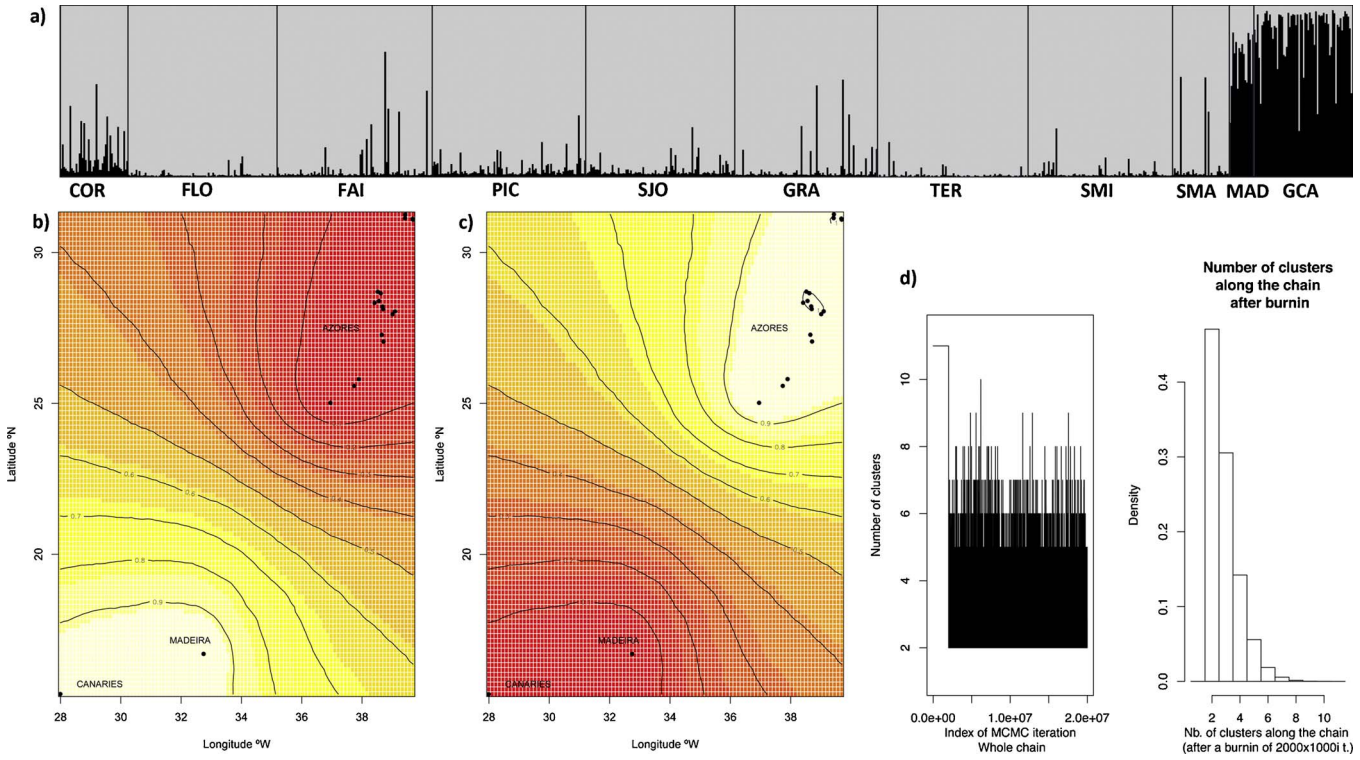


Fig. 3. Bayesian analysis results for *Patella aspera* using 11 loci following: a) STRUCTURE clustering analysis considering admixture and prior information for population origin; bar graph show the average probability of membership (y-axis) of individuals (n = 841, x-axis) in K = 2 clusters (see Fig. 1 for population codes); b, c) GENELAND spatial analysis showing the assignment of individuals to clusters for K = 1 and K = 2, respectively; darker and lighter shading are proportional to posterior probabilities of membership in each cluster, with lighter (yellow) areas showing the highest probabilities of clusters; d) posterior density distribution of the number of clusters (clear mode at K = 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the literature (see Garza and Williamson, 2001), increased the detection of bottlenecks at higher values of the pre-bottleneck Θ (Fig. B5). Estimates of contemporary migration rates calculated with BAYESASS showed no signs of gene flow between clusters (Table B6). Results of bottleneck and migration analyses did not differ when using a reduced loci dataset, *i.e.* only loci with < 20% proportion of null alleles.

4. Discussion

Even though a high dispersal potential is expected in *P. aspera* as drawn from the pelagic larval duration of its congeneric European continental form *P. ulyssiponensis,* which is estimated to range between 14.5–27 days (average age of planktonic life *in vitro* after metamorphic competence; see Ribeiro, 2008), unfavourable oceanographic conditions, large larvae mortality and the relatively large geographical distance among populations may have prompted the isolation between Azores and those on the remainder of the Macaronesia archipelagos. In fact, larval transport may be strongly affected by local eddies, current reversals and other unknown oceanographic physical patterns (Sponaugle et al., 2002; Palumbi, 2004). Moreover, marine larvae often

Table 2
Genetic variation observed at eleven microsatellite loci within two clusters of *P. aspera* identified by STRUCTURE and GENELAND analyses. The "null free" inbreeding coefficient $F_{IS\text{-}INEST}$ for within cluster is highlighted in bold.

Cluster/Locus	ASP2	ASP3	ASP7	ASP17	ASP21	ASP27	ASP29	ASP33	ASP36	ASP38	ASP39	ALL
AZORES												
Na	15	10	11	14	17	19	39	20	22	20	13	
$A_R(12)$	5.0	4.1	2.2	5.1	5.2	4.8	6.5	5.9	5.9	4.5	2.8	4.7
$A_P(12)$	1.4	1.2	1.2	2.1	2.1	1.2	3.8	3.0	3.0	2.0	1.3	2.0
H_O	0.611	0.200	0.085	0.292	0.559	0.323	0.253	0.428	0.304	0.252	0.162	0.315
H_E	0.795	0.589	0.509	0.774	0.784	0.774	0.836	0.773	0.811	0.701	0.363	0.701
F_{IS}	0.232	0.660	0.834	0.620	0.289	0.581	0.702	0.449	0.626	0.638	0.555	0.399
Null	0.10	0.25	0.28	0.27	0.13	0.25	0.28	0.20	0.28	0.27	0.18	
MADEIRA + CANARIES												
Na	12	9	3	9	12	11	21	19	12	11	3	
$A_R(12)$	6.7	5.4	7.0	4.3	4.2	4.4	6.7	7.1	5.2	5.1	1.9	5.3
$A_P(12)$	3.2	2.4	5.9	1.4	1.1	0.8	4.0	4.2	2.2	2.6	0.4	2.6
H_O	0.123	0.098	0.000	0.230	0.437	0.197	0.310	0.557	0.409	0.349	0.091	0.285
H_E	0.818	0.802	0.370	0.653	0.695	0.782	0.784	0.876	0.808	0.745	0.292	0.697
F_{IS}	0.876	0.869	0.833	0.676	0.287	0.791	0.627	0.359	0.475	0.514	0.792	**0.104**
Null	0.40	0.39	0.37	0.27	0.12	0.35	0.28	0.15	0.20	0.22	0.17	

Na = number of alleles; $A_R(g)$ = allelic richness (*g* accounts for the maximum standardized sample size *i.e.* twice the number of genotypes); $A_P(g)$ = private allelic richness; H_O = observed heterozygosity; H_E = unbiased expected heterozygosity; F_{IS} = uncorrected inbreeding coefficient; *Null* = frequency of null alleles.

face a huge mortality over relatively short distances from their spawning locations (*e.g.* Cowen et al., 2000). Larvae biological traits (*e.g.* growth, swimming and orientation capacities, reproductive and recruitment strategies) and species interactions (*e.g.* predation, food availability) may also determine a limited connectivity within a given species (Cowen and Sponaugle, 2009). In fact, genetic breaks in marine invertebrates with high putative dispersal capacity are not uncommon in the literature (see Galarza et al., 2009; Serrano et al., 2014; Ougajjou and Presa, 2015; Guiñez et al., 2016). The relatively fragile nature of patellid larvae (when compared, for instance, with crustacean larvae), the realized larval pelagic duration which is presumably shorter in the field (*e.g.* Olson and McPherson, 1987) and the predominately oligotrophic character of NE Atlantic waters surrounding the Macaronesia archipelagos (Marañon et al., 2000; Silva et al., 2013), may act to favour local retention and limit the dispersal capacity of *P. aspera* over large distances. Thus, genetic differentiation in *P. aspera* probably arose from the historical and contemporary complex interplay of particular biological and physical processes that favoured isolation over population homogeneity. Nevertheless, the shortest distance between Madeira and Canaries and the presence of the Selvagens islands in between both archipelagos, are likely to facilitate larvae dispersal and population connectivity of *P. aspera* among those archipelagos.

High inbreeding coefficients were detected in all populations. Such large deviations from HWE and excess of homozygotes are often strongly linked to the occurrence of null alleles across loci, with most population genetic studies avoiding their use in analyses. However, deviations from HWE can also be biological meaningful, especially when such deviations occur at multiple loci, and may be explained by several nonexclusive factors such as inbreeding, selection against heterozygotes and the Wahlund effect (Dakin and Avise, 2004; Allendorf and Luikart, 2007). The individual inbreeding model implemented in INEST showed that, although null alleles have an undoubted role on heterozygote deficit and interpopulation differentiation, inbreeding appears as the most likely process underpinning the observed positive F_{IS} values. Processes that lead to inbreeding in natural populations (*e.g.* non-random mating) are often considered to be more effective and associated with small populations and would hardly affect marine populations with expected high census population sizes. However, stochastic factors acting upon the recruitment success and especially the anthropogenic influence on the population dynamics and demography can lead to an unbalanced genetic composition in widespread marine populations (see O'Leary et al., 2013; Aglieri et al., 2014). Such processes can result in unexpected levels of relatedness among individuals and therefore inbreeding rates higher than expected. Exploitation of *P. aspera* across Macaronesia not only has reduced its abundance, but also the mean size of individuals (see *Implications for the management and conservation* below). Since *P. aspera* is a protandric hermaphrodite, therefore undergoing sex change from male into female as they grow, harvesting is strongly biased towards females. Such reduction in the number of females is believed to trigger non-random mating and the production of individuals which relatedness is higher than expected by chance. In such over-harvested populations, the census size (*N*) can thus greatly differ from the effective population size (*Ne*), where the number of individuals that actively contributes for the next generation being rather small. In fact, a reduction in *Ne* would counter the mechanisms that promote random mating in *P. aspera*, such as high fecundity, external fertilization with broadcast spawning, and putative extensive larval dispersal. Moreover, the fact that harvesting is size selective towards the larger individuals may impose a strong selection among heritable characteristics within exploited populations (see Conover and Munch, 2002). Noteworthy, recent research has revealed that the sex-ratio of *P. aspera* remained unchanged regardless the increasing fishing pressure, suggesting that males are compensating for the removal of larger females by undergoing sex change at smaller and presumably earlier sizes (Martins et al., 2017), a pattern that has also been observed in other patellid limpets (Borges et al., 2016). Although this mechanism can buffer populations against changes in sex-ratio of the species, the fact that females became smaller than their historical size, would affect the amount of gametes released into the water, with obvious negative consequences for the reproductive output of *P. aspera*. Moreover, it is still undetermined how this compensatory mechanism is to affect the gametic fitness of such small-size females or its impact on larval viability and dispersal capacity. Under these circumstances, inbreeding can emerge from a large variation in individual reproductive success influenced by particular environmental, reproductive and behaviour factors, with only a few individual limpets reproducing and actively contributing to the genetic pool of the next generation (see Hedgecock, 1994). Although inbreeding is suggested as the main driver for the observed excess of homozygotes in *P. aspera* populations, other alternative mechanisms such as extensive within-population genetic structure (*i.e.* the Wahlund effect), higher rates of molecular evolution and randomly induced differences in allele frequencies between sperm and eggs have also been proposed to explain deviations from HWE in free-spawning marine invertebrates (see Addison and Hart, 2005). In fact, the occurrence of a Wahlund effect cannot be entirely discarded, as unrecognized temporal breeding subunits may occur inside sampled populations, resulting in large variations from HWE. Moreover, seasonal or inter-annual changes in currents might bring together larvae from locally differentiated sources, providing the baseline for genetic patchiness and spatial substructuring of within-population demes. Yet, a Wahlund effect is less likely in our study, since each local geographic sample was tested separately and Bayesian clustering analyses did not detect any within-sample structuring, or within archipelago for that matter (*i.e.* Azores). Preliminary analyses for within-island genetic variation (two sampled locations within each island) also showed the absence of any population structuring at such smaller spatial scale. Furthermore, the impact of the Wahlund effect is usually more substantial when cryptic populations, recipient and donor, exhibit large differences in gene diversity (Zhivotovsky, 2015); in this case, independently of distance, all *P. aspera* populations are relatively genetic impoverished with similar diversity, have individuals with alike morphological traits and there are no apparent differentiated niches where putative cohorts could genetically thrive distinctively. For instance, Corte-Real et al. (1992) found no genetic differences between intertidal and subtidal specimens of *P. aspera* in the Azores. More importantly, although homozygote excess was shown to be surprisingly common in free-spawning species (see Addison and Hart, 2005), deviations to HWE should be, above all, interpreted under the life-cycle particularities and circumstances of a given species. As so, given the acceptable sample size and number of microsatellite markers used in this study, the genome-wide effect detected (*i.e.* significant heterozygote deficits across all loci screened in all populations studied), the specificities of *P. aspera* reproductive/breeding cycle and the unbalanced nature of harvesting, inbreeding seems the most plausible mechanism for the observed excess of homozygotes in *P. aspera* populations from Macaronesia. The retention of planktonic larvae near parent habitats, which is not uncommon even in species with very high dispersal potential (*e.g.* Swearer et al., 1999), may further support the idea that there are no high levels of immigration from other sites that otherwise would reduce the chances of inbreeding. A deeper insight into the spatial genetic structure of *P. aspera* populations would be possible by taking the so-called 'seascape genetics' approach, and use oceanographic modeling (*e.g.* Galindo et al., 2006) as well as many other interacting forces and traits to understand the processes involved in marine connectivity of the species (see review by Selkoe et al., 2016)

Studies that include the effect of overexploitation or a reduction in the effective population size on the genetic diversity and inbreeding phenomena of marine invertebrates are fairly scarce (*e.g.* Gallardo et al., 1998; Constantini et al., 2007), and examples are often provided for other organisms such as fishes (*e.g.* Hutchinson et al., 2003; Hoarau et al., 2005; O'Leary et al., 2013). The generalized idea is that inbreeding, although occurring naturally in many populations, is

intensified by overexploitation reducing the capability for adaptation and increasing the extinction risk (Keller and Waller, 2002; Frankham, 2005). Under these circumstances, exploited populations will tend to suffer to a greater extent from demographic and genetic stochasticity and face higher chances of extinction due to inbreeding or loss of genetic diversity by drift (Frankham et al., 2014). Population subdivision can also foster inbreeding by decreasing migrants among subpopulations and prompting chances for mating among relatives (Bierne et al., 1998; Andrade and Solferini, 2007). In such case, and even assuming random mating, inbreeding could occur because population sizes are very limited and the consequences of genetic drift become apparent (Keller and Waller, 2002). In fact, population differentiation in *P. aspera* given by pairwise distances estimates (F_{ST} and D_{est}) indicated some degree of subdivision among islands (see Table 2). These estimates, however, may be unreliable, and interpretation should be cautious due to the known impact of null alleles in overestimating population differentiation. With much greater support, our findings suggested that i) Azores samples comprise an isolated population and ii) Madeira and Canaries populations cluster together in a single group. Moreover, insular populations of *P. aspera* are thought to have been isolated from continental forms for the last 4–8 Ma (-Pinto et al., 2005, 2008; -Pinto et al., 2005, 2008). These same studies, using mtDNA analyses, revealed genetic differentiation between Azores and the remaining archipelagos. Given the high of isolation and the similarity in environmental conditions among archipelagos, genetic diversity is not expected to be high. Whereas genetic diversity, under these circumstances, can be lost purely due to genetic drift, the prolonged and long-time harvesting of limpets across archipelagos is likely to have negatively impacted genetic diversity and favoured inbreeding due to a reduction in the effective population size in *P. aspera*. Unfortunately, there are no current estimates of *P. aspera* effective population sizes throughout the region. Yet, considering the historical and more recent Catch Per Unit Effort (CPUE) as a reliable proxy of population census sizes in *P. aspera*, *i.e.* in the early 1990's there were still places in Azores where CPUE was around 12 kg/half hour but this has fallen to 5 g/half hour in recent years (OSPAR-Commission (2010), it is clear that a dramatic reduction in the abundance of animals has taken place, which is likely to have negatively affected the present-day *Ne* in *P. aspera* across the region.

4.1. Implications for the management and conservation

Harvesting of *P. aspera*, which is currently listed in the OSPAR list of threatened and/or declining species and habitats (OSPAR-Commission, 2010), is under specific legislation throughout the Macaronesia region. At each archipelago, seasonal fishing closures and minimum catch sizes have been imposed: Azores (October–April, 50 mm); Madeira (December–March, 40 mm); and Canaries (December–April, 45 mm). Moreover, harvesting is forbidden inside specific marine protected areas and/or limpet protection zones in each archipelago. Catch limits (kg) per day for commercial (with permit only) and recreational use have also been established. Seasonal closures have broadly been defined in agreement with reproductive periods occurring during colder months (see Vale, 2016). Despite this, non-compliance with current legislation is the norm among limpet harvesters (see Diogo et al., 2016). Empirical evidence suggests that *P. aspera* stocks are pretty much depleted and overexploited (Martins et al., 2008, 2011) and the lack of enforcement of regulations is seen as the most likely reason for the failure to protect limpet populations. To raise awareness, regional authorities should promote environmental education actions directed to fishermen and general public, for the importance of the sustainable use of limpets as a resource as well as a key ecological asset. Limpet protection zones, which have been shown to be largely ineffective (see Martins et al., 2011; Diogo et al., 2016), would likely work if permanently monitored and surveyed by local authorities. Management approaches should also target intermediate-sized individuals by establishing an upper size limit on limpets (Connover and Munch, 2002; Allendorf et al., 2008). In the short term, this would increase the number of larger females potentially generating higher-quality offspring and contributing to an increasing effective population size in *P. aspera* (see Birkeland and Dayton, 2005) and thus helping reducing inbreeding rates. From a stock perspective, and considering the population differentiation found among the Azorean and other archipelagos' populations, both Madeira and Canaries authorities should join efforts in establishing a common management strategy for the protection and harvesting of *P. aspera* in those archipelagos. Moreover, the high levels of isolation and exploitation of the Azorean populations suggests that authorities must act immediately to avoid the genetic and demographic depletion of *P. aspera* stocks in the Azores. Scientifically sound data on this limpet reproductive cycle, its recruitment rate and its demographic status would help inform conservation actions and formal stock definition. Although genetic approaches are only a small piece in the intricate puzzle of defining effective fishery stocks, this study suggests that limpet conservation and fisheries management across Macaronesia should, at the very least, consider two independent stocks: one in Azores and another one in Madeira − Canaries. Management and conservation initiatives need to be revisited in the light of such results. Above all, the most direct way to reduce the effects of over-exploitation on *P. aspera* across the Macaronesian region is to drastically reduce the intensity of harvest of its populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fishres.2017.09.003.

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