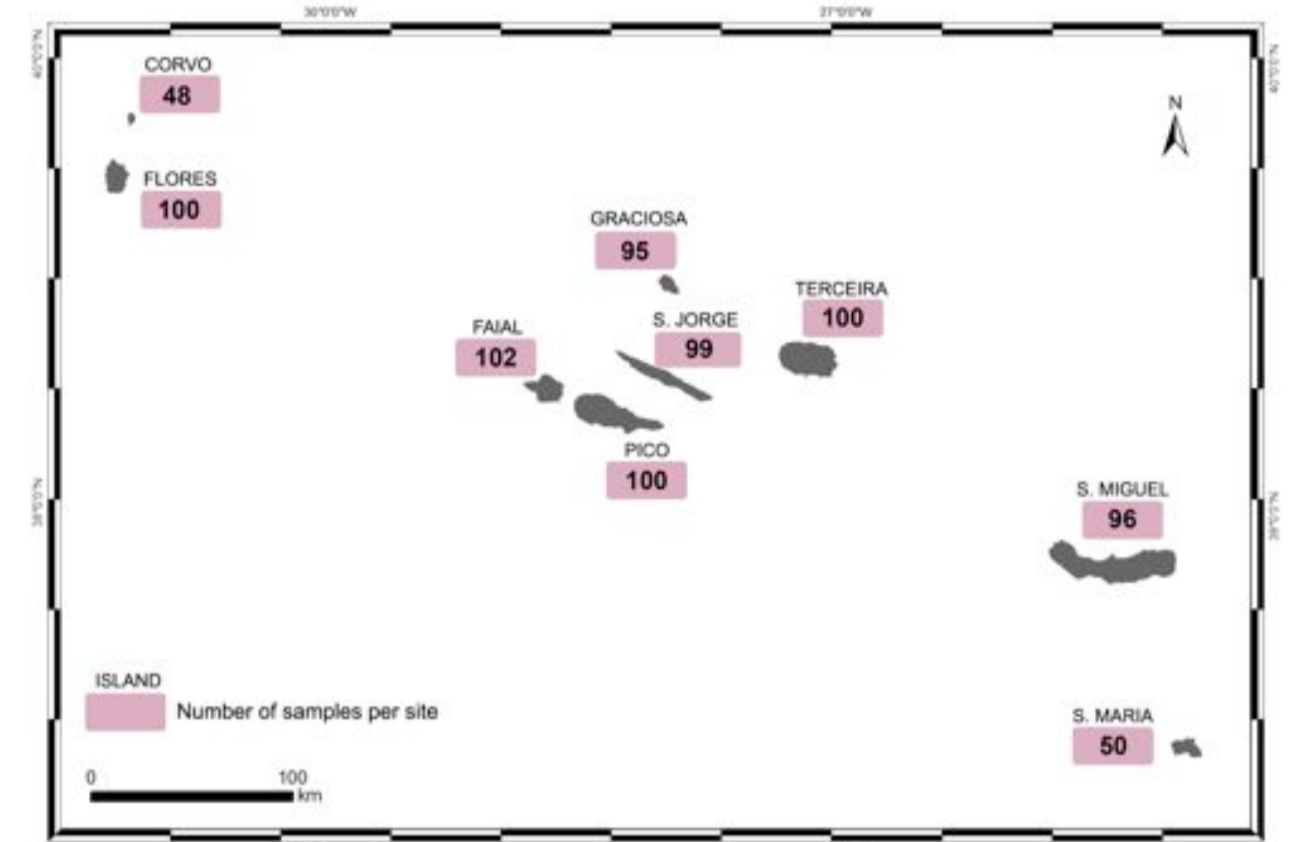


GENETIC POPULATION STRUCTURE AND CONNECTIVITY OF AZOREAN LIMPETS

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ABSTRACT There is growing consensus that anthropogenic activities significantly disrupt the structure and functioning of marine ecosystems. Oceanic islands such as the Azores (NE Atlantic) are unique habitats with fragile communities, which are highly susceptible to degradation and ecosystem disruption. Patellid limpets have traditionally been collected as a food resource and in 1988 the limpet fishery in São Miguel Island collapsed, after which a one-year ban was implemented allowing the stocks to recover and avoiding catastrophic overexploitation effects. In 1993, legislation was passed to protect this resource, i.e. limpet no-take areas were created, seasonal harvesting restrictions were applied and minimum legal catch sizes were established. However, a recent survey has shown that limpet populations still show clear signs of overexploitation and some populations are virtually extinct in some islands. Here we have developed new multiplexed and described microsatellite markers for the species *Patella aspera* and *P. candei* and have examined their genetic diversity, gene flow and population connectivity in the Azores Archipelago. Overall, such information is a fundamental asset to inform conservation strategies and to promote the sustainable exploitation of Macaronesian limpets.

METHODS

- Novel microsatellite markers were described for *P. aspera* and *P. candei* using 454 next-generation sequencing.
- A total of 790 of *P. aspera* were collected in Azores (all islands). Additional samples were taken from Canary islands (n=30) and mainland Europe (n=49) (the later identified as the most recent ancestral species *P. ulysiponensis*). Similarly, 902 samples of *P. candei* were obtained from all islands in Azores (these samples are still being genotyped, so results will only be referred to *P. aspera*).
- Genotypes of all samples of *P. aspera* for 17 microsatellites were scored using GENEMAPPER™ v.4.2 (Applied Biosystems).
- Allele frequencies and observed (H_o) and expected heterozygosity (H_e) were estimated in GenAlEx (Peakall and Smouse 2006). Allelic richness [Ar(g)] were estimated using the rarefaction method implemented in ADZE (Szpiech et al. 2008). Linkage disequilibrium, inbreeding coefficients (F_{IS}) and deviations from the Hardy-Weinberg equilibrium (HWE) were tested in GENEPOP (Raymond and Rousset 1995).
- The presence and frequency of null alleles was tested for each locus using MICROCHECKER (van Oosterhout et al. 2004). The IIM approach (individual inbreeding model) was used in INEST (Chybicki and Burczyk 2009) to partition out the influence of null alleles on F_{IS} values.
- Unbiased F_{ST} values were obtained using FREENA (Chapuis and Estoup 2007). Population structure was also analyzed using the Bayesian model-based clustering approach implemented in STRUCTURE (Pritchard et al., 2000), using prior population information. Selection of the most likely number of genetic clusters (K) was based on the Evanno et al. (2005) method.
- Analysis of molecular variance (AMOVA) was used to partition total molecular variance using ARLEQUIN (Excoffier et al. 1992), on groups identified by STRUCTURE analysis.



Patella aspera (RÖDING, 1798)

Distribution: Azores, Madeira, Selvagens, the Canaries, and North of Africa.
Habitat: Rocky shores, lower intertidal and sublittoral zones.
Description: The conical shell is light in colour and structurally strong, exhibiting thick and irregular margins. The underside of the muscular foot is yellowish.
Reproduction: *P. aspera* is a protandrous species. Organisms start off as male but many change to female as they age. This means that there are two types of males, permanent males and temporary (protandric) ones.



Patella candei (d'ORBIGNY, 1839)

Distribution: Endemic to Macaronesia archipelagos with different subspecies
Habitat: Rocky shores, intertidal and shallow sublittoral zones.
Description: The conical dark shell has regular margins, and is smoother and thinner than *P. aspera*. The underside of the muscular body (foot) on which the limpet moves around is dark grey.
Reproduction: *P. candei* is a gonochoric species, meaning that individuals do not change sex throughout their lives. Spawning occurs around the year but it is thought to peak during Autumn/Winter.

RESULTS

Table 1. Multilocus genetic diversity for *Patella aspera*.

Multilocus	CORVO	FLORES	FAIAL	PICO	SJORGE	GRACIOSA	TERCEIRA	SMIGUEL	SMARIA	CANARIAS	LISBOA	All populations
N	48	100	102	100	99	95	100	96	50	30	49	669
Mean Ar(g)	10.480	10.260	10.169	10.221	10.163	10.193	10.029	10.046	9.824	NA	NA	10.154
Mean H_o	0.494	0.470	0.447	0.447	0.452	0.462	0.486	0.484	0.468	0.323	0.305	0.440
Mean H_e	0.783	0.743	0.759	0.745	0.758	0.755	0.750	0.758	0.748	0.671	0.663	0.739
Avg(F_{IS})	0.514	0.056	0.037	0.149	0.064	0.048	0.210	0.052	0.143	0.263	0.098	0.083

N = number of samples; Ar(g) = allelic richness (g accounts for the maximum standardized sample size i.e. twice the number of genotypes); H_o = observed heterozygosity; H_e = unbiased expected heterozygosity; unbiased F_{IS} = inbreeding coefficient. Significant departure from Hardy-Weinberg equilibrium after Bonferroni correction is shown in bold.



Table 2. Pairwise estimates of unbiased F_{ST} among all populations sampled for *Patella spp.*

	CORVO	FLORES	FAIAL	PICO	SJORGE	GRACIOSA	TERCEIRA	SMIGUEL	SMARIA	CANARIAS	LISBOA
CORVO	-										
FLORES	0.004	-									
FAIAL	0.002	0.005	-								
PICO	0.006	0.001	0.002	-							
SJORGE	0.005	0.003	0.003	0.002	-						
GRACIOSA	0.005	0.003	0.002	0.001	0.000	-					
TERCEIRA	0.007	0.003	0.003	0.002	0.002	0.000	-				
SMIGUEL	0.001	0.004	0.001	0.002	0.003	0.004	0.002	-			
SMARIA	0.005	0.010	0.014	0.009	0.014	0.013	0.011	0.010	-		
CANARIAS	0.050	0.054	0.050	0.051	0.048	0.046	0.052	0.050	0.056	-	
LISBOA	0.196	0.125	0.109	0.117	0.112	0.113	0.115	0.111	0.115	0.129	-

Bold values are significant after sequential Bonferroni correction.

Table 3. Analysis of molecular variance (AMOVA) for K=2.

Source of variation	Variation (%)	F-statistics	P-value
Among groups (K=2)	14.55	F_{CT} : 0.153	<0.000
Among populations within groups	0.74	F_{SC} : 0.008	<0.000
Within populations	84.71	F_{ST} : 0.146	<0.000

Table 4. STRUCTURE analysis in *P. aspera* for K=2 (most likely K under Evanno's method)



CONCLUSIONS

- Genetic diversity was affected by the presence of null alleles in many of the loci and populations tested (Table 1); Yet, unbiased F_{IS} values show that homozygote excess may be a consequence of actual inbreeding between individuals and populations. In fact, it is thought that only a relatively small reproductive population size exists in the Azores, as a consequence of the high levels of human-exploitation.
- No evidence of genetic differentiation among islands in the archipelago of Azores was found for *P. aspera* (Table 2; Table 3).
- As expected, and also supported by previous studies using mtDNA (Sá-Pinto et al. 2008), *P. aspera* from the islands is separated from its close-related species *P. ulysiponensis* of the mainland (LISBOA at ~1400km), with some loci failing to amplify correctly (Table 2; Table 3; Table 4). *P. ulysiponensis* is known to be the most-recent ancestral of *P. aspera*.
- Although unbiased pairwise F_{ST} estimates support a small differentiation between Azores populations and Canarias, the STRUCTURE analysis did not provide the same result. When excluding *P. ulysiponensis* from the analysis, STRUCTURE reveals differentiation between Azores and Canarias populations, as supported by the unbiased F_{ST} estimates, which would be expected due to the geographical distance between archipelagos (~2200km).

ONGOING RESEARCH

- This study is part of an ongoing research project focusing on the conservation of limpet species in the Azores (www.patelgene.com), but also including other exploited patellids from the Macaronesian archipelagos (Canarias, Madeira and Cape Verde).
- A similar approach is also being used for the *P. candei* complex (ongoing genotyping), a species showing a high level of morphological diversity among and within the Macaronesian archipelagos.
- Spatial and temporal patterns of limpet recruitment are also being examined in parallel.

