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Disentangling the Influence of Mutation and Migration in Clonal Seagrasses Using the Genetic Diversity Spectrum for Microsatellites

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Abstract:

The recurrent lack of isolation by distance reported at regional scale in seagrass species was recently suggested to stem from stochastic events of large-scale dispersal. We explored the usefulness of phylogenetic information contained in microsatellite loci to test this hypothesis by using the Genetic Diversity Spectrum (GDS) on databases containing, respectively, 7 and 9 microsatellites genotypes for 1541 sampling units of *Posidonia oceanica* and 1647 of *Cymodocea nodosa*. The simultaneous increase of microsatellite and geographic distances that emerges reveals a coherent pattern of isolation by distance in contrast to the chaotic pattern previously described using allele frequencies, in particular, for the long-lived *P. oceanica*. These results suggest that the lack of isolation by distance, rather than the resulting from rare events of large-scale dispersal, reflects at least for some species a stronger influence of mutation over migration at the scale of the distribution range. The global distribution of genetic polymorphism may, therefore, result predominantly from ancient events of step-by-step (re)colonization followed by local recruitment and clonal growth, rather than contemporary gene flow. The analysis of GDS appears useful to unravel the evolutionary forces influencing the dynamics and evolution at distinct temporal and spatial scales by accounting for phylogenetic information borne by microsatellites, under an appropriate mutation model. This finding adds nuance to the generalization of the influence of large-scale dispersal on the dynamics of seagrasses.

Keywords: Population structure and phylogeography ; Conservation genetics and biodiversity ; Clonality ; Genetic Diversity Spectrum ; genetic divergence ; microsatellites ; seagrass ; stepwise mutation

1. Introduction

The limited dispersal of propagules and gametes across heterogeneous environments is a key component of genetic differentiation in space and time, and is considered to be a first step toward speciation, addressed in the early model of Isolation by Distance (Wright, 1943). The Isolation by Distance (IBD) model continues to provide a framework to analyze and interpret the genetic structure of species across spatial scales (Epperson, 1990; Epperson and Li, 1996; Fenster, Vekemans and Hardy, 2003; Planes*, et al.*, 2002; Rousset, 1997; Slatkin, 1992; Vekemans and Hardy, 2004). Indeed, understanding the scale-dependence of genetic variability within a species range allows inferences on dispersal scales as well as on the influence and strength of the evolutionary processes generating spatial segregation of polymorphism.

Many species providing and shaping habitats on land and in the seas are clonal, including a variety of trees and grasses, and all seagrass and coral species. Recently, this life history trait has been associated with the observation of low divergence rates or even lack of polymorphism across long regions of nuclear or organellar DNA among congeneric species, and lack of polymorphism within species even at the scale of the entire species range (Aires*, et al.*, 2011; Olsen*, et al.*, 2004). This is thought to result from the long life spans of clonal lineages and overlapping generations potentially resulting in slow evolutionary rates (Aires*, et al.*, 2011; Arnaud-Haond*, et al.*, 2012). These peculiarities imply that conventional phylogeographic approaches (Alberto*, et al.*, 2008; Arnaud-Haond*, et al.*, 2007; Coyer*, et al.*, 2004; Olsen*, et al.*, 2004; Serra*, et al.*, 2010) based on gene diversification have limited power to retrace the large scale biogeographical events shaping the distribution of polymorphism in these species.

The genetic distance spectrum (GDS), an individual-based approach summarizing the scale-dependent structure of genetic diversity within a seagrass meadow, allows us to infer the role of several processes (clonality, somatic mutation, selfing, outcrossing and immigration) in shaping the genetic composition within populations (Moalic*, et al.*, 2011; Rozenfeld*, et al.*, 2007). In practice, the GDS is represented as the frequency distribution of genetic distances among individuals based on microsatellite markers (Rozenfeld*, et al.*, 2007) and adapted from inter-population distances previously proposed (Goldstein*, et al.*, 1995; Slatkin, 1995). The GDS illustrates the distribution of the size differences between microsatellite motifs (alleles) similar to the “mismatch distributions” commonly drawn on the basis of gene sequences (Harpending, 1994). Thus, provided that the mutation model does not depart significantly from the underlying hypothesis of the Stepwise Mutation Model (SMM), the examination of the GDS across a broad geographic range shows the variable weight of these processes in shaping the genetic structure of individual populations. Analysis of the GDS across seagrass meadows revealed the prevalence of clonality, as well as immigration from distinct meadows, albeit at low frequency (Rozenfeld*, et al.*, 2007). Immigration was inferred by the detection of individuals exhibiting a distance to other members of the same deme that largely exceeded the average outcrossing distance, even simulated over a high number of outcrossing generations within the deme (Rozenfeld*, et al.*, 2007). Comparison of the within population GDS of two sister brown algal species with contrasting reproductive modes (dioecious *versus* selfing hermaphroditic) revealed their clearly different mating systems (Moalic*, et al.*, 2011).

Although the GDS has been used to explore the balance of the evolutionary forces that yield the genetic composition of populations and the mating system within seagrass meadows or algal beds, the concept of the GDS could be extended to explore the genetic structure of

species across their entire biogeographic range, allowing examination of the scale-dependent weight of the different processes involved.

Here, we extend the individual GDS proposed at the within-population scale by Rozenfeld et al. (2007), to examine processes occurring along the entire biogeographic range. We examine the distribution of genetic distances for two clonal species of seagrass with contrasting mating systems, the long-lived monoecious *Posidonia oceanica* and the dioecious, shorter lived, *Cymodocea nodosa*, across their entire biogeographic range. Previous analyses of both species showed high spatial genetic structure with geographical clustering of populations along their distribution range and differentiation of meadows at all spatial scales. Despite this, genetic and geographic distances were not correlated, particularly at regional (i.e. within basins) and local scales (within regions). Lack of Isolation by Distance (IBD) at local and regional scales may be explained by two contrasting scenarios. First, effective space colonization may lead to random drift by preventing subsequent effective migration even at reduced spatial scales (Becheler*, et al.*, 2010). Second, occasional large scale dispersal events may explain the general lack of IBD reported in most studies, as suggested by a recent meta-analysis of seagrass population genetics studies (Kendrick*, et al.*, 2012). In the first case it is expected that, provided the analyzed microsatellites predominantly evolve through SMM, the phylogeographic information contained by microsatellites may help retracing past events of colonization or gene flow (Hardy*, et al.*, 2003). In such scenario, the GDS based on phylogenetic distance would be expected to show a simultaneous increase of genetic and geographic distance. Contrastingly, if long distance migration events are the main cause, the lack of IBD previously reported on the basis of allelic frequency would persist in a GDS analysis. Also, since the *a priori* delineation of populations, panmictic units or biogeographic provinces is not an easy task in clonal organisms (Becheler*, et al.*, 2014; Becheler*, et al.*, 2010), an inter-individual approach –rather than an a priori grouping of individuals in sampling locations- may be a required and informative step towards providing objective spatial or ecological delineation. Here, we aimed to i) describe the evolution of inter-individual genetic distances with increasing geographic distances. We examine the accuracy of using phylogenetic information contained in microsatellite size to provide information on scales of differentiation (despite their still-debated mode of evolution and the drawbacks due to possible homoplasy during long-term divergence processes). ii) Assess the information that a GDS snapshot can yield about the balance among evolutionary forces acting at different spatial scales.

2. Material & Methods.

2.1. Model species

Posidonia oceanica is a clonal marine angiosperm restricted to the Mediterranean Sea, where it is an essential structural species, developing extensive meadows ranging from 0 to 40 m in depth. It is a very slow-growing organism, with the clones extending horizontally through the growth of rhizomes at approximately 2 cm year^{-1}, developing shoots (ramets, the individual module repeated by clonal propagation) at intervals of approximately 5–10 cm. This monecious species (i.e., both male and female flowers on the same plant) is characterized by rarely observed episodes of sexual reproduction (Diaz-Almela*, et al.*, 2006), important clonality (Arnaud-Haond*, et al.*, 2007) and extremely long clonal life spans (Arnaud-Haond*, et al.*, 2012). Shoots are attached through tough rhizomatic connections and although some exceptional settlement of drifting shoots has been occasionally observed in very particular patch recruitment conditions, no such event was detected in established meadows despite extensive surveys (Diaz-Almela*, et al.*, 2008). These characteristics

suggest rare migration among *Posidonia oceanica* meadows, as confirmed by population genetics analysis (Arnaud-Haond*, et al.*, 2007), despite the positive buoyancy of seeds. Individual *P. oceanica* shoots (ramets) live for up to 50 years, and clones have been aged at several thousand years (Arnaud-Haond*, et al.*, 2012).

Cymodocea nodosa is also a clonal, but faster growing and shorter-lived, marine angiosperm distributed both in the Mediterranean and in the Eastern Atlantic, from Senegal to Central Portugal and to the Canaries and Madeira islands. It can be found from very shallow water to depths in excess of 30 m, forming rather dense and large meadows at depth. *C. nodosa* reproduces regularly in meadows containing a mixture of males and females, but reproduction cannot be completed in monoclonal stands dominated by a single clone (either male or female), as is the case along European Atlantic meadows dominated by single males (Alberto*, et al.*, 2008). The seeds are formed at the base of the shoots of females, and exhibit negative buoyancy, suggesting rather limited dispersal (Marba and Duarte, 1995), and confirmed by genetic spatial autocorrelation (Alberto*, et al.*, 2005). However, the occurrence of isolated meadows around oceanic islands shows the ability for occasional large scale dispersal through the transport of either seeds or, more likely, drifting shoots possibly carrying seeds.

2.2. Sampling and genotyping

The datasets analyzed here were originally developed to study the biogeography of both *P. oceanica* (Arnaud-Haond*, et al.*, 2007) and *C. nodosa* (Alberto*, et al.*, 2008)across their biogeographic range. Approximately 40 shoots were sampled in each of 37 localities for *P. oceanica*, ranging from west to east, from the Spanish Mediterranean Coast to Cyprus, encompassing a distance range of 3500 to 4000 km. A similar sampling was conducted for each of 49 meadows of *C. nodosa*, including the Canaries and Madeira archipelagos and extending along the species range in the Atlantic continental African and European coasts as well as encompassing the entire Mediterranean basin to the Eastern Mediterranean (Cyprus). In all meadows, shoots were collected at randomly drawn coordinates across an area of 20 m x 80 m and 60 m x 14 m for *P. oceanica* and *C. nodosa*, respectively (see Arnaud-Haond *et al.*, 2007 and Alberto *et al.*, 2008 for additional details). Both datasets are archived and available (http://ifisc.uib-csic.es/EDEN/output.php#data).

Specimen collection consisted of a portion of the meristem of each shoot that was desiccated and preserved in silica crystals. Genomic DNA was isolated following a standard CTAB extraction procedure (Doyle and Doyle, 1987). Two sets of seven and nine microsatellite markers were used for *P. oceanica* and *C. nodosa*, respectively (Alberto*, et al.*, 2003). Both sets of markers were previously determined to yield the most cost-effective determination of clonal membership (Arnaud-Haond*, et al.*, 2005; Arnaud-Haond*, et al.*, 2007). These included loci Po4-3, Po5, Po5-10, Po5-39, Po5-40, Po5-49 and Po 15 for *P. oceanica* and Cn2–86, Cn2–38, Cn2–14, Cn2–16, Cn2–18, Cn4–29, Cn2–45, Cn2–24 and Cn4–19 for *C. nodosa*. All loci were perfect dinucleotides, except Cn18-2 that was a compound microsatellite composed of three dinucleotide motifs. Finally, each shoot or ramet is characterized by a series of pairs of microsatellite alleles at k loci with k=7 for *P. oceanica* and k=9 for *C. nodosa.* Ramets derived from the same original seed (i.e., belonging to the same genet or clone) will be represented by the same allele combination except for possible somatic mutations occurring during vegetative growth.

2.3. Genetic Diversity Spectrum

The Genetic Diversity Spectrum was constructed from the frequency distribution of all pairwise inter-individual genetic distances for each species, using perl scripts available on request. We used the so-called Rozenfeld"s distance (Moalic*, et al.*, 2011; Rozenfeld*, et al.*, 2007), a distance metric designed to illustrate the effect of clonality and, in contrast with previously available metrics, helps to resolve individuals' origin at an earlier time. This measure of similarity among sampling units yields a distance of zero between identical genotypes (Rozenfeld*, et al.*, 2007).

The genotype of a particular sampling unit, called *A*, is represented as:

$$A = (a_1, A_1)(a_2, A_2)\ldots(a_k, A_k),$$

where a_i and A_i are the allele lengths (in number of nucleotides) of locus *i* in each of the two (diploid) chromosomes for *k* loci. Lower case represents the smaller allele in the pair.

Given a second sampling unit, *B*, with genotype

$$B = (b_1, B_1)(b_2, B_2)\ldots(b_k, B_k),$$

we define a dissimilarity degree between *A* and *B* at locus *i* as:

$$d_i(A,B) = \min\left(|A_i - B_i| + |a_i - b_i|, |A_i - b_i| + |a_i - B_i|\right),$$

providing a parsimonious (i.e., minimal) representation of the genetic distance, understood as the difference in allele length, between sampling units *A* and *B*. The *genetic distance* among sampling units is then defined by summing the contributions from each locus (Rozenfeld*, et al.*, 2007):

$$D(A,B) = \sum_{i=1}^{k} d_i,$$

providing the degree of global dissimilarity between *A* and *B (Rozenfeld distance)*. According to this definition, sampling units from genetically identical individuals (genets or clones) will have a distance of zero.

The distribution of genetic distance D between sampling units is represented as a frequency distribution of all pairwise values, which is referred to as the Genetic Diversity Spectrum (GDS, Rozenfeld*, et al.*, 2007). The GDS is analogous to the frequency distribution of pairwise differences used on some clonal organism to analyze the effect of clonality and reveal possible somatic mutations or scoring errors (Arnaud-Haond*, et al.*, 2007; Douhovnikoff and Dodd, 2003; Meirmans*, et al.*, 2003; Van der Hulst*, et al.*, 2003).

A second set of GDS was also built for comparative purpose by using the distance based on Shared Alleles (SAD, Chakraborty and Jin, 1993) based on the proportion of the genome shared, independently of the length differences among microsatellites alleles.

2.4. Genetic Diversity Spectrum of random communities

We tested the shape of the GDS against two null hypotheses: i) panmixia and ii) genetic structure without footprint of colonization and migration history on the spatial distribution of microsatellites (no phylogenetic information borne by microsatellites).

i. Panmixia, implemented *via* a randomly drawn distribution: Under the hypothesis of global panmixia, the shape and width of the GDS would depend only on the number and level of polymorphism of the markers used and on their random recombination. Panmixia, as a random association of alleles across genotypes by locus, was simulated for both species across their entire range by randomly picking the observed alleles (at observed frequencies) to reconstruct genotypes. In order to take into account the occurrence of clonality in our dataset and compare real data with their random equivalent, some of the random panmictic genotypes were chosen within each simulated population to be replicated in the "random dataset" the same number of times as the repeated MLG were detected in the same population in the "natural dataset".

ii. Lack of footprint of migration, implemented via a Structured Allele Shuffling method: As departure from panmixia is observed through several analysis including previous *F*st or Bayesian based reports (Arnaud-Haond et al., 2007; Alberto et al., 2008), we more precisely aimed to test the departure of GDS from that expected under the hypothesis of a lack of phylogeographic footprint on the spatial distribution of allelic divergence at microsatellite loci. In order to do so we randomly shuffled allele sizes for each locus and assigned each of the alleles to another one. A brief example: example for a locus with three possible alleles of lengths 154,156 and 160, a random reshuffling may lead to reallocate length 160 instead of 154, 154 instead 156 and 156 instead of 160. This was generated by a language dictionary that we used to "translate" all genotypes. This Structured Alleles Shuffling maintained the genotypic and genetic differentiation of population based on allelic identity (but not length) and allele frequencies as well as clonality. In this way the phylogenetic information borne by microsatellite size is randomly distributed among samples, allowing specific tests for the significance of its relation to geographic distance in the original dataset.

As a result, in both cases, simulations were made at the ramet level constraining the number of replicated genotypes in each location to that observed in the real datasets in order to compare real and random or shuffled distributions with the same amount of clonality.

This resulted in the construction of matrices of pairwise distances and GDS for randomly distributed alleles across space. The significance of the deviations between observed and permuted (obtained by simulations under the two null hypotheses tested) GDS was tested by Kolmogorov-Smirnov tests in R.

The same test was performed, for comparative proposes, using the Shared Allele Distance and assessing the departure of GDS from that obtained under the null hypothesis of panmixia.

2.5. Scale-dependence of the Genetic Diversity Spectra

In order to examine the scale-dependence of the GDS, it was dissected into multiple GDS"s by sequentially selecting, from the overall matrix used to construct the GDS, all pairwise distances for individuals sampled across increasing ranges of Euclidean geographic distances. In particular, the classes used were 0 km (same locations), 100 km (0km<distances<=100km), 500 km (100km<distances<=500km), 1000 km (500km<distances<=1000km), 2000 km (1000km<distances<=2000km), 3000 km (2000km<distances<=3000km), 4000 km (3000km<distances<=4000km) and >4000km. These distances were chosen arbitrarily since the number of genetic distance values inside each class is higher than 30000.

For each species, a Mantel test was then performed for both species and compared assuming dispersal in one dimension by plotting genetic *versus* geographic distance and in two dimensions, by plotting genetic versus log (geographic distance).

3. Results

A total of 1541 and 1647 ramets derived from 39 and 60 different meadows were used to build the Spectrum of Genetic Distances of *P. oceanica* and *C. nodosa* across their biogeographic ranges, resulting in 2373140 and 2710962 pairwise distances that ranged from 0 (for clone mates) to 51 for *P. oceanica* and 31 for *C. nodosa* using Rozenfeld distance. The observed GDS"s significantly deviate from the random simulated distribution (Fig. 1), showing a significant departure from random association of polymorphism among Multi Locus Genotypes (MLG) across the species distribution ranges for *P. oceanica* distribution (K-S D = 0.40, p<0.001) but not for *C. nodosa* (K-S D = 0.10, p = 0.99). Similarly, the departure of shuffled allele distribution (Fig.1) from the real one was significant for *P. oceanica* (K-S D = 0.31, p<0.001) but not for *C. nodosa* (K-S D = 0.26, p = 0.15).

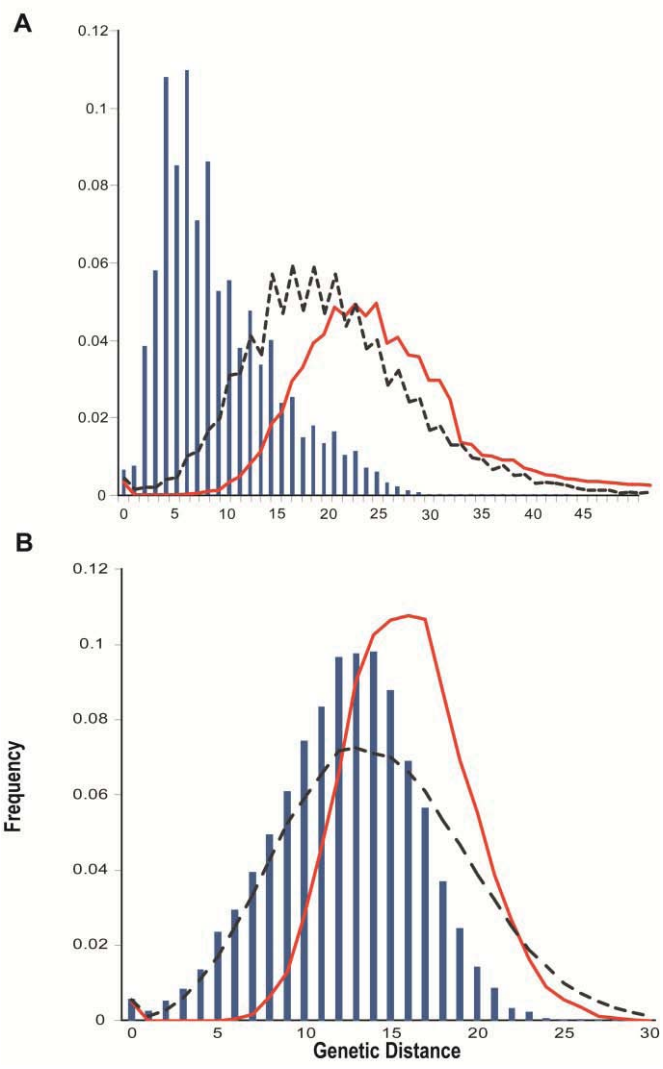

Figure 1 : Genetic Distance Spectra (GDS, blue bar plots) representing the frequency distribution of Rozenfeld inter-individual distances at microsatellites (based on microsatellite allele size differences) for *Posidonia oceanica* (A) and *Cymodocea nodosa* (B) across their geographic ranges: from East to West Mediterranean, and from East Mediterranean to East Atlantic, respectively. Red lines represent the randomly drawn distributions and black dashed lines, the distributions after random shuffling of allele sizes. In both permutations approaches, permuted dataset were constrained in order to maintain the same level of clonality (i.e. the same amount of repeated MLG) in each population identical to that in the original dataset. Kolmogorov–Smirnov tests were significant for both random permutations and allelic shuffling for *P. oceanica* (D = 0.40, p<0.001 and D = 0.31, p<0.001, respectively), but for none of the tests for *C. nodosa* (D = 0.10, p = 0.99 and D = 0.26, p = 0.15, respectively).

The GDS of both species start at 0 (Fig. 1), illustrating the prevalence of clonality. A low frequency of the smaller genetic distance classes was also observed, likely to represent the occurrence of scoring errors, somatic mutations, or both (Arnaud-Haond*, et al.*, 2007; Rozenfeld*, et al.*, 2007). Apart from this, the GDS"s were fundamentally different for both species, with slow growing and high longevity *P. oceanica* showing a GDS skewed towards lower genetic distances and composed of several sub-peaks, compared to the almost perfect bell-shape distribution of the spectrum for *C. nodosa* (Fig. 1). Contrastingly, the" GDSs based on Shared Allele Distance were both strictly unimodal (Fig. S1). The GDS was significantly departing from the random ones for *C. nodosa* (K-S D=0.47, p=0.046) but slightly below significance for *P. oceanica* despite similar departure of the curves (K-S D=0.47, p=0.076), therefore possibly due to limited statistical power.

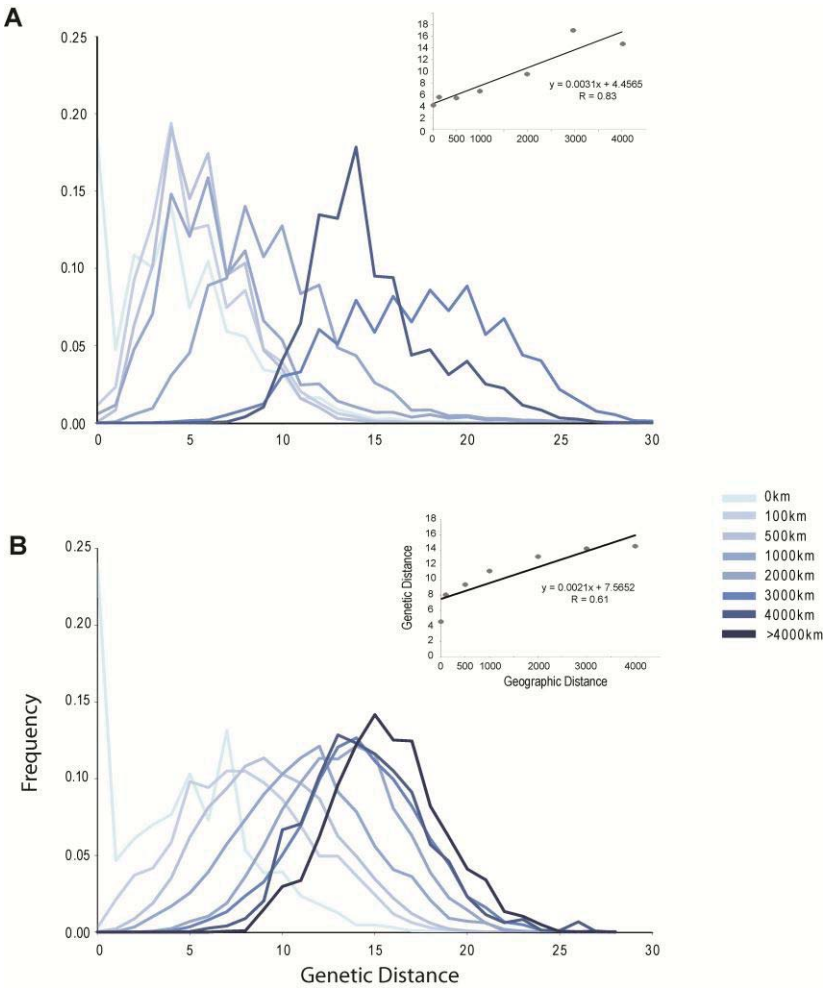

Figure 2: Decomposition of the Genetic Distance Spectra across distinct sets of geographic distances, illustrating the change in mean Rozenfeld genetic distances (based on microsatellite allele size differences) with geographic distances for *Posidonia oceanica* (A), and for *Cymodocea nodosa* (B). The insets show the regression corresponding to the significant Mantel tests performed on bins of geographic distances (Table 1).

The scale-dependence of the GDS based on Rozenfeld distance was examined by decomposing the GDS into partial GDS"s derived from comparisons of pairwise distances of ramets within increasing distance ranges (Fig. 2). This exercise further demonstrates important differences between the GDS"s of *P. oceanica* and *C. nodosa*. Although most partial GDSs are overlapping, there is a clear linear increase in average genetic distance with increasing geographic distance in *P. oceanica* (Fig. 2A). The larger pair-wise genetic distance classes were dominated by pairs of ramets located at distant locations across the

Mediterranean basin. The same tendency, but with a much narrower shift in genetic *versus* spatial distance, was obtained for *C. nodosa,* where partial GDS's show a greater overlap (Fig. 2B). Mantel tests based on bins of those sequential GDS were highly significant (Table 1) for both *P. oceanica* ($R=0.83$ and $R=0.79$ $p<0.001$ in one and two-dimension spaces, respectively) and *C. nodosa* ($R=0.61$ and $R=0.48$ $p<0.01$ in one and two-dimension spaces, respectively). Both analyses confirm the scale-dependence of the genetic structure for both species in a pattern closer to a one dimension dispersal model, and its greater strength in *Posidonia oceanica*.

The dissection of GDS based on SAD also showed an evolution of genetic distance with geographic ones (Fig S2). However this was true only up to a certain point as the peaks at large geographic distance are largely overlapping. Mantel tests showed significant but less strong relationships (Table 1), with the exception that for *C. nodosa* no significant relationship was detected in a two-dimension scheme.

Table 1: Results of the Mantel tests for both species with Rozenfeld and Allele Shared distances plotted against geographic distances or their log values to fit a one dimension (1D) or two-dimension (2D) model. Significance of Mantel tests are indicated with one to three asterisk for $p<10^{-2}$ to $p<10^{-4}$.

	Posidonia		Cymodocea	
	1D	2D	1D	2D
Rozenfeld distance	0.83**	0.79**	0.61*	0.48*
Allele Shared distance	0.75**	0.62***	0.46*	0.32

4. Discussion

Our GDS analysis opens new perspectives concerning the main evolutionary forces affecting the ecology and evolution of seagrasses, emphasizing their mating system. The recurrent lack of overall Isolation By Distance at the regional scale reported for several seagrass species, including *P. oceanica* (Arnaud-Haond et al., 2007) and *C. nodosa* (Alberto et al., 2008) was recently proposed to stem from occasional events of stochastic large scale dispersal (Kendrick*, et al.*, 2012). This led the authors to suggest the importance of large scale dispersal for the ecology and evolution of those species. Results presented here offer an alternative or complementary hypothesis by revealing the existence of an IBD pattern observed when taking into account the phylogenetic information borne by microsatellites. The results indicate the dominant role of mutation-drift *versus* a negligible influence of contemporary effective migration. Such a pattern may result from the strong influence of historical colonization patterns, followed by local recruitment and accumulation of divergent mutations through drift, and implies low dispersal and strong influence of clonality on the dynamics and evolution of some seagrass species. Differences in the patterns observed between both species also suggest the influence of life history traits and/or history on the weight of these distinct scenarios.

The Genetic Distance Spectrum (GDS) of the two seagrass species examined here indeed departed significantly from randomness with Rozenfeld distance only for *P. oceanica*, and with SAD only for *C. nodosa*, revealing distinct but significant patterns of genetic structure across their distributional ranges. This departure was particularly pronounced for *Posidonia oceanica*, which exhibited successive peaks in the Rozenfeld based GDS (Figure 1A),

revealing a system of populations sub-structured into divergent clusters of individuals, despite the lack of IBD previously reported on the basis of F_{ST} (Arnaud-Haond*, et al.*, 2007). In contrast, the GDS for *Cymodocea nodosa* showed a profile typical of a normal distribution (except for the clonality appearing at 0) with a single mode at high genetic distances. This reveals a lack of hierarchical divergence (Figure 1B), even though differentiation occurs, as revealed by the departure from randomness observed for the SAD-based GDS and Mantel tests using partial GDS either based on Rozenfeld or SAD distances. Indeed, for both species, decomposing the GDSs into partial GDS"s at increasing spatial scales (Fig. 2) revealed regular and positive relationships between genetic and geographic distances, reflecting dispersal limitation at small spatial scales, and the history of divergence of clones across their distribution ranges at larger scales. This corresponds to an isolation by distance model applicable even at the intra basin scale (Fig. 2, Fig. S2, Table 1). This pattern contrasts with the lack of evidence for intra-basin IBD (within marine regions bordered by the Siculo-Tunisian or the Gibraltar straits) reported for the same populations on the basis of F_{ST} differentiation levels, for *P. oceanica* (Arnaud-Haond*, et al.*, 2007) and *C. nodosa* (Alberto*, et al.*, 2008), where the only indices of a relationship between geographic and genetic distance emerging at global scale was due to biogeographic breaks.

The increasing genetic divergence with spatial distance revealed by the partial GDS"s provides evidence of isolation by distance (IBD), and reveals very low effective dispersal in these species. The lack of correlation between high genetic differentiation and geographic distances at regional scale observed in previous F_{ST} based studies was proposed to be the result of the strong influence of genetic drift and stochastic migration of sexually produced propagules, with little influence of geographic distance on the success of dispersal events (as suggested in Kendrick*, et al.*, 2012). However, F_{ST} estimates based on the frequency of alleles in a strongly structured system, as in the present case, may soon saturate the maximum level of divergence that can be revealed, resulting in an asymptotic value of the curve describing the increase of F_{ST} with the divergence time (Hutchison and Templeton, 1999). In such a saturated profile, only very slight differences, possibly lower than the standard error of the estimates, are expected among the mean F_{ST} located beyond the asymptote threshold. This is accompanied by a variability in the point of equilibrium mutation-migration-drift depending on the markers and models used. Such saturation of frequency-based distances using hypervariable markers impedes the accurate discrimination of distinct levels of differentiation beyond a certain threshold, which may have been overcome in the case of highly differentiated meadows. This threshold is highly dependent on the variability of the markers used (Beaumont and Pether, 1996; Hedrick, 1999; Jost, 2007; Jost, 2008), with more variable markers usually leading to earlier saturation. This phenomenon may be responsible for the apparent inconsistency between GDS and F_{ST} analyses, with regard to the relationship between genetic and geographic distances at the regional scale. Indeed, it becomes a challenge to distinguish high levels of differentiation from even higher ones on the basis of F_{ST} estimates prone to a non-negligible variance beyond a certain threshold, contrary to the observed increase reported here for partial GDSs (Fig 2). Some corrections have been proposed using distance metrics based on allele frequency to obtain estimates that would not depend on the level of variability within demes, such as the recently proposed D or G"st (Hedrick, 1999; Hedrick, 2005; Jost, 2007; Jost, 2008). The main condition for the reliable performance of these modified metrics seems to be a relatively higher influence of mutation versus migration on the mutation-migration-drift equilibrium specific to study the systems in question. Results obtained here (Fig 2) suggest that these conditions are fulfilled in the two seagrass species studied, but additionally support the existence of relevant phylogeographic information in microsatellites at least for the long-lived *P. oceanica*.

The geographic coherence of the divergence observed here from the information contained in microsatellites across species distributional ranges indeed confirms that, under a relatively higher influence of mutation *versus* migration on the mutation-migration-drift equilibrium, an additional benefit may be obtained by using not only the allelic frequencies, but also the

potential phylogenetic information contained in the difference in number of motifs borne by distinct alleles (Goldstein*, et al.*, 1995; Goldstein and Pollock, 1997; Slatkin, 1995; Takezaki and Nei, 1996). Estimates based on allelic frequencies alone assume an Infinite Allele Model (IAM), with mutation giving rise to a new allelic state uncorrelated with the mutated allele, while there is evidence that this model is not accurate for most microsatellite loci (Schlotterer, 2000). The use of differences in microsatellite length can therefore reveal phylogeographic patterns given an underlying mutation model (Stepwise Mutation Model, SMM) possibly closer to that actually operating on these markers (Balloux and Goudet, 2002; Goodman, 1997; Hardy*, et al.*, 2003; Rousset, 1996). Compared with the estimators D or G"st, the GDS approach used here accounts for the high level of diversity, while simultaneously taking advantage of meaningful information conveyed by microsatellites alleles that depends on the relative influence of mutation in the mutation-migration-drift equilibrium, and also on the accuracy of the mutation model assumed, here the SMM.

The analysis of partial GDS"s presented here confirms the very strong structure revealed by F_{ST} for both species, but also reveals coherence between spatial and genetic distances that remained hidden in previous analyses that ignored the phylogenetic information contained in microsatellite length variation. The species studied here have contrasting reproductive modes and dispersal biology; fruits of *P. oceanica* remain buoyant over several weeks and have a rather high dispersal potential (Balestri and Lardicci, 2008), while seeds of *C. nodosa* are negatively buoyant and remain attached to the mother plants until germination. On the other hand, ramets of *C. nodosa* are more likely to fragment and provide a mid-range dispersal mean than the much thicker rhizomes of *P. oceanica*. Yet despite their biological differences, reflected in different GDS patterns, both species show a comparable high spatial genetic structure, demonstrating that the dispersal potential is not fully realized for either of them. This limitation seems particularly important in *P. oceanica* where not only the Mantel tests but also the GDS itself shows departure from random expectations, suggesting an even stronger influence of mutation and a greater limitation to gene flow in this long-lived species than in *C. nodosa*. The correlation between genetic divergence and geographic distance reported here may result from the high influence of mutation, rather than contemporary migration, on the pattern of spatial distribution of polymorphism among populations. This holds even at the regional scale as this shift in the distribution of genetic distances is already evident at scales of several hundreds of kilometers (Fig. 2). Extant meadows may, therefore, be the result of ancient stepwise colonization events, followed by a rather allopatric evolution with very few migration events and a dominant clonal space occupation. The initial pattern induced by the colonization process is thus preserved and mostly determined by distance to the source. Such a scenario is supported by the sparse flowering (Diaz-Almela*, et al.*, 2006; Diaz-Almela*, et al.*, 2009) and very low sexual output observed for *P. oceanica* (Balestri and Cinelli, 2003; Balestri, Piazzi and Cinelli, 1998; Diaz-Almela*, et al.*, 2006; Diaz-Almela*, et al.*, 2009) across the Mediterranean, in line with the limited (Arnaud-Haond*, et al.*, 2005; Arnaud-Haond*, et al.*, 2007) though still likely overestimated (Arnaud-Haond*, et al.*, 2007) clonal diversity in *P. oceanica* meadows. It is also in line with extremely low expected dispersal linked to the negative buoyancy of seeds in *C. nodosa*, and strikingly contrasted patterns of genotypic diversity in neighboring meadows for both species (Alberto*, et al.*, 2006; Alberto*, et al.*, 2008; Arnaud-Haond*, et al.*, 2007). This initial colonization behavior followed by predominantly local and clonal recruitment was proposed by Eriksson (1993) to be one of the extreme possible life history strategies for clonal plants, referred to as Initial Seedling Recruitment (ISR) as opposed to Repeated Seedling Recruitment (RSR) involving the occurrence of periodical events of seed recruitment. Such an ISR pattern was diagnosed for several seagrass species on the basis of spatial autocorrelation analysis (Alberto*, et al.*, 2005; Becheler*, et al.*, 2010) and is equivalent to the persistent imprint of initial founder effects described in non-clonal populations followed by self-recruitment over millennia (e.g., Neiva et al. 2012). The ISR seems to imprint genetic composition over large time scales as the divergence observed in line with geographic distances suggest that the population system would be „crystallized" since the last (re)colonization of present day meadows,

resulting in a genetic structure mostly driven by clonal growth, competition and drift after a 'stepping-stone' colonization process. This would also be in agreement with recent findings of extreme life span for some *P. oceanica* clones(Arnaud-Haond*, et al.*, 2012), the domination of vast areas by single clones of *C. nodosa* (Alberto*, et al.*, 2008; Alberto, Mata and Santos, 2001) and reports of extreme generation time for clonal organisms (Laberge, Payette and Bousquet, 2000; Oinonen, 1969; van der Merwe, Spain and Rossetto, 2010; Vasek, 1980). There is a peak inversion between the two last distance bins in *P. oceanica* (Figure 2 and to a lower extent Fig. S2) which seems to contradict the general interpretations given before and based on geographic distance alone. This change in ordering is mostly due to an inversion of distance among meadows sampled in Greece and Cyprus compared to those present in Central or Western part of the Mediterranean. This may either be attributed to historical pathways of migration or (re) colonization among Mediterranean basins, or to differential effects of mutation and drift in the populations in those two regions.

Finally, such an ISR strategy has been shown to result in genetic differentiation at all geographic scales the seagrass meadows studied, including among neighboring quadrates within meadows, rendering the genetic concept of population cumbersome (Becheler*, et al.*, 2010). This challenges the *a priori* delineation of sets of samples to be pooled or not, to perform summary statistics, while the use of the GDS to identify scales of differentiation does not require such *a priori* delineation and can help in recognizing the breaks corresponding to the distance below which genetic structure is mostly due to ISR and consequent genetic patchiness, and above which divergence occurs as a result of mutation accumulation through longer term isolation. This threshold appears to occur beyond a few hundred kilometers for both species (Fig. 2, Fig 3).

This work shows that, when microsatellites exhibit a mutation model close enough to stepwise, the Genetic Distance Spectrum (GDS) analysis is a useful complementary tool to analyze the scale dependence of evolutionary processes and unravel the biogeography of some species across a large part of their biogeographic range. This is particularly useful for clonal species for which many other approaches fail due to the exceedingly low evolution of genes classically used for phylogeographic reconstruction. While showing that useful phylogenetic information may also be borne by microsatellites, as previously emphasized (Goldstein*, et al.*, 1995; Rousset, 1996; Slatkin, 1995), the evidence presented here also support the prerequisite of a predominant importance of mutation to use allele length at microsatellites in a phylogenetic framework.

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Supplementary Figures

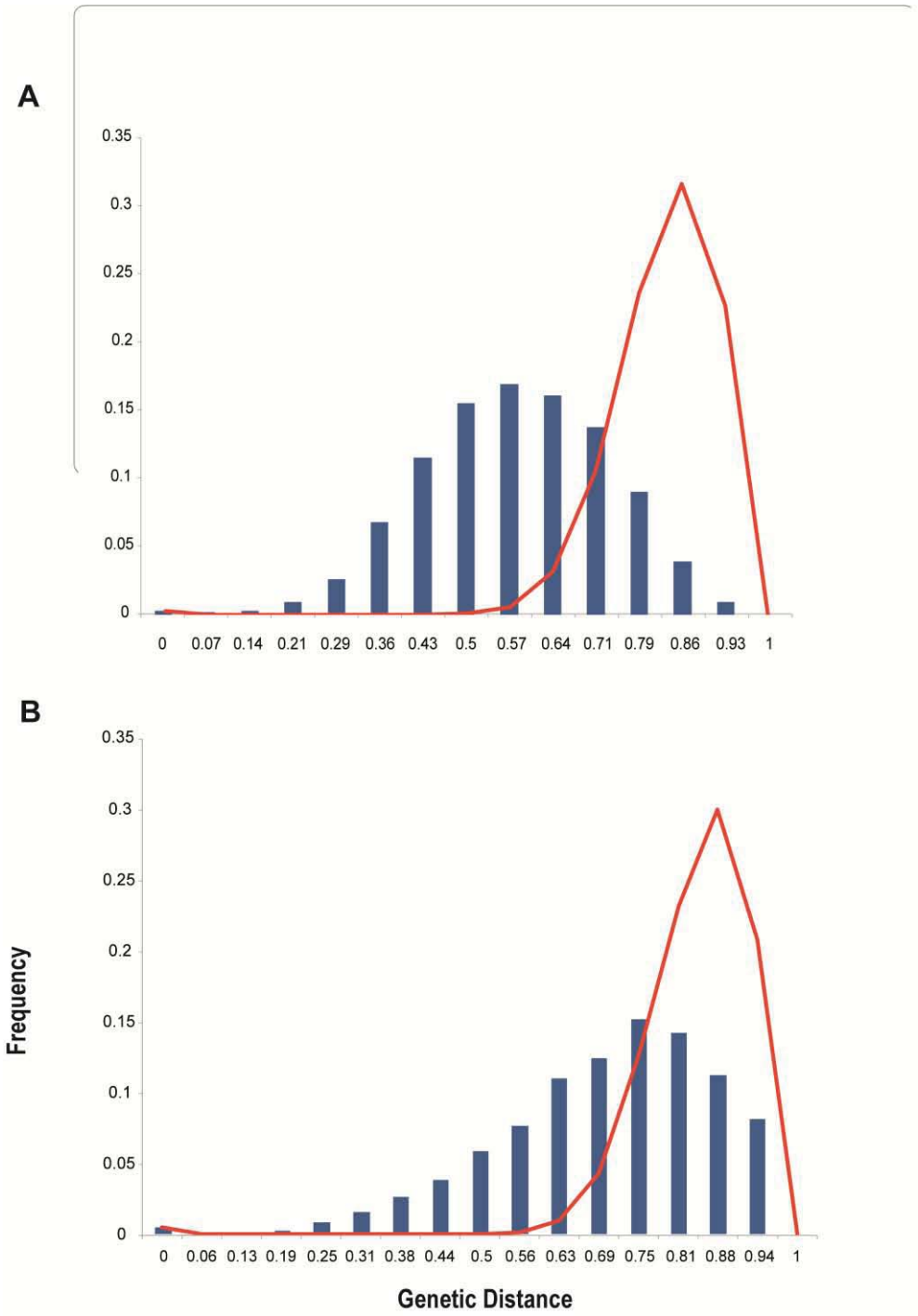

Figure S1 : Genetic Distance Spectra (GDS) representing the inter-individual Shared Allele Distances (SAD) at microsatellites for *Posidonia oceanica* (A) and *Cymodocea nodosa* (B) across their geographic ranges: from East to West Mediterranean, and from East Mediterranean to East Atlantic, respectively. The red lines show the shape of the expected spectrum under the hypothesis of panmixia.

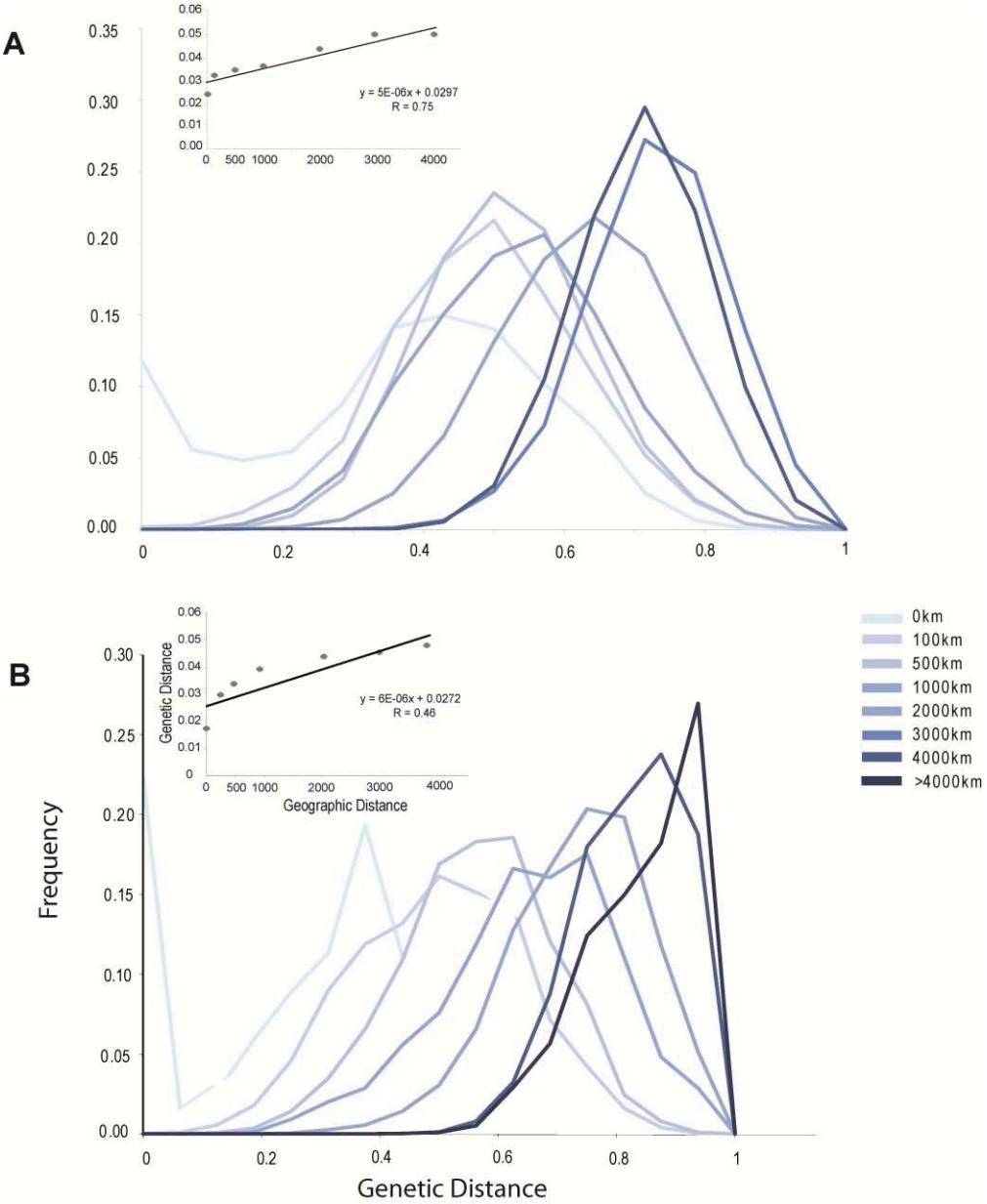

Figure S2: Decomposition of the Genetic Distance Spectra across distinct sets of geographic distances, illustrating the change in mean Shared Allele Distance (SAD) with geographic distances for *Posidonia oceanica* (A), and for *Cymodocea nodosa* (B).