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**BIOGEOGRAPHIC PATTERNS OF MEDITERRANEAN
AMPHIPODS ASSOCIATED WITH *POSIDONIA OCEANICA*
MEADOWS**

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1. INTRODUCTION

1.1 The research framework: patterns of diversity in the Mediterranean Sea

The Mediterranean Sea, enclosed between Europe, Africa and Asia is connected to the Atlantic Ocean through the Strait of Gibraltar in the west, while is connected to the Sea of Marmara through the Straits of Dardanelles and to the Black Sea in the east. In the southeastern part, the Suez Canal links the Mediterranean Sea to the Red Sea and Indian Ocean (Coll et al., 2010). The climate of Mediterranean area is characterized by warm and dry summers and wet and cold winters; as a result, the temperature of the basin is characterized by a strong seasonality and by a remarkable East to West and North to South gradient (Danovaro et al., 1999). Accordingly, the main ecological features of the Mediterranean Sea clinally vary. The average salinity (about 36.2 ppt) tends to increase eastward, generating a gradient with a maximum of 38.0-38.05 ppt in the easternmost part of the basin due to the intense water evaporation (Patarnello et al., 2007). The decrease in primary production from North to South and from West to East is inversely proportional to the increase in temperature and salinity (Coll et al. 2010). The basin is generally oligotrophic and exhibit strong environmental gradients for this feature too, with the eastern part more oligotrophic than the western, which is considered ultra-oligotrophic (Danovaro et al., 1999; Krom et al., 2005).

A latitudinal gradient of species richness decreasing from north-eastern to south-western regions has been detected in the Mediterranean Sea, correlated with clines of both environmental variables and production levels, together with general water mass circulation and the location of barriers (Coll et al., 2010; Lejeusne et al., 2010). The closeness to Alboran Sea is also considered responsible of the higher number of species in the Western part. The Central Mediterranean, Adriatic and Aegean present intermediate levels of species richness, while the Adriatic Sea displays a lower species number due its isolation, peculiar water circulation, limited exchange with the western basin and the presence of massive freshwater discharge (Dimentman, 2006). The eastern Mediterranean and the Levantine basin have in general the lowest species richness due to extreme environmental conditions that may be prohibitive for temperate species, such a high temperature and salinity (Coll

et al., 2010). According to environmental features, the Western basin hosts more cold-temperate species and shows similarities with Atlantic Ocean faunas, while the Eastern basin hosts species of sub-tropical origin and shares part of its fauna with Indian Ocean (Lejeusne et al., 2009).

All these ecological and biological features allowed identifying biogeographic sectors within the Mediterranean Sea, each characterized by both different biota and ecological parameters: 1) Alboran Sea, 2) North Western Mediterranean, 3) Tyrrhenian Sea, 4) Adriatic Sea, 5) Ionian Sea, 6) Aegean Sea and 7) Levantine basin (Bianchi & Morri 2000).

Despite the Mediterranean Sea covers a surface area of 0.82% and 0.32% of the volume of world's ocean, the amount of species inhabiting the basin represent 4-18% of the world's marine species, approximately 17000 (Coll et al., 2010; Patarnello et al., 2007). A high rate of endemism characterizes its biota, averaging more than one-quarter of the resident species (Lejeusne et al., 2009). This high species richness and rate of endemism make the basin a "biodiversity hot-spot". One reason of this great diversity in species had its roots in the complex geological history of the basin, which led to multiple colonization events from different oceans associated with repeated and dramatic environmental change, causing speciation through vicariance and local adaptation (Bianchi & Morri, 2000; Lejeusne et al. 2009).

Indeed, the Mediterranean Sea is what remains of a much larger ocean, the Tethys, that during the Cretaceous, after the opening of the Atlantic Ocean, was connected to the newborn Indian Ocean through an uninterrupted equatorial belt. At this stage, Tethys was colonized by a highly diversified fauna of warm-tropical origin. During the Oligocene (about 30 Mya) a shrinking of the Tethys Ocean and the decrease in warm water supply produced colder conditions and an associated mass extinction, followed by the formation of the isthmus of Suez during the Miocene that separated the Indo-Pacific Ocean from the Mediterranean (Bianchi & Morri, 2000). For this reason, the actual Mediterranean species of warm-tropical origin are considered as remnants. About 6 Mya, in late Miocene, the inflow of Atlantic water was interrupted and the Mediterranean became an isolated sea. Drastic climate changes caused sea level to drop and salinity to rise across the entire basin, an event that took the name of Messinian Salinity Crisis (MSC) (Maldonado, 1985; Garcia-Castellanos et al., 2009). The negative balance of water supply led to the almost complete desiccation of the basin that reduced to a series of large evaporitic lakes (Bianchi & Morri, 2000). The desiccation of the Mediterranean could

have brought the biota of Tethys nearly to extinction: this could be particularly true for deep fauna (Buochet & Taviani, 1992), while probably a small part of the surface water fauna could have survived in areas of refuge (Stanley, 1990; Bellan-Santini et al., 1992; Myers, 1996). With the reopening of the Strait of Gibraltar at the beginning of the Pliocene, waters of the Atlantic poured into the Mediterranean basin allowing the dispersion of marine biota between the Atlantic and the Mediterranean (Almada et al., 2001; Domingues et al., 2005). Alternation between glacial eras and warm interglacial periods during the Quaternary has produced various migratory waves of Atlantic fauna (Bianchi et al. 2000). As a result of this complex geological history, the present Mediterranean marine biota is composed by species from different biogeographic categories and although it is derived primarily from Atlantic Ocean, the vast range of climates and hydrology has contributed to the co-occurrence and survival of both temperate and sub-tropical organisms (Sarà *et al.*, 1985; Bianchi & Morri., 2000).

1.2 The study environment: *Posidonia oceanica* meadows

The seagrass *Posidonia oceanica* (L.) Delile is a marine endemic phanerogam widely distributed in the Mediterranean Sea, where it represents the dominant seagrass species (Bourdouresque et al., 2006). It is a superior plant belonging to Posidoniaceae (subphylum Angiospermae, class Monocothyledonae) with differentiated vegetative structure, similar to terrestrial plants, as roots, rhizome (modified stem), leaves and a sexual reproduction with flowering. *P. oceanica* is a stenoeic species that does not grow where salinity is lower than 33‰ and in conditions of water turbidity. As a consequence, the bathymetric distribution is influenced by light penetration and is variable between less than 1 m to a maximum of 40 m. The plant and its formations (the meadows) are well represented all along the Mediterranean coastal areas, although in the Western part of the basin it disappears in correspondence to Almeria-Oran front, where superficial Atlantic Ocean waters enter in the basin and *P. oceanica* does not meet the optimal conditions to grow (Gobert et al. 2006). In the Eastern basin it disappears along the Egyptian coastal waters, in correspondence to Nilo Delta and in the area of Israel, Palestine and Lebanon (Mitchel 2011). Except for these areas, *P. oceanica* meadows distribution is continuous all along the basin where it forms monospecific large meadows, or sometimes in association with other phanerogams like *Cymodocea nodosa* or seaweeds like *Caulerpa prolifera*.

This peculiar ecosystem is considered one of the most valuable, in terms of goods and services provided, in the Mediterranean Sea for its key ecological roles: habitat engineering, oxygen production and prevention of coastal erosion through energy dissipation due to wave breaking (Boudouresque et al., 2006; Ondiviela 2014). This formation has a complex and well structured architecture and is characterized by a high biodiversity in terms of both plant and animal communities (Buia et al., 2000). More than 400 species spend their entire lives in *P. oceanica* meadows so making them a “biodiversity hotspot”. Moreover, *P. oceanica* ecosystem plays other fundamental functions, such as providing nursery habitat or refuge from predators and represents a direct and indirect source of food for a great number of organisms (Boudouresque et al., 2006; Mazzella et al., 1992). Although being protected and listed among priority habitats (1120*) in the Habitat Directive (92/43/EEC), the meadows are experiencing an extensive regression due to anthropogenic disturbance caused by a number of local factors such as urbanization, illegal fishing, aquaculture and pollution besides global impacts like climate change and biological invasions. *P. oceanica* is particularly affected by environmental quality conditions and become sparse, up to disappear, when the pollution *sensu lato* increases. For this reason the meadows are considered good bioindicators of coastal waters quality (Pergent et al., 1995; Montefalcone, 2009).

Knowledge about genetic diversity of *P. oceanica* populations may help not only to understand the actual status of the resource but also to plan restoration actions (Procaccini et al., 2012). For this reason, many authors focussed on genetic structure of *P. oceanica* populations. The present patterns of genetic diversity and population distribution of *P. oceanica* are mainly lead by its paleogeographic history, that is affected by the dramatic changes occurred in the Mediterranean basin during the past geological Eras. Several studies highlighted a West-East separation in the genetic structure of *P. oceanica* populations, as observed for other species in the Mediterranean biota (Ignatiades et al., 2009; Gambi et al., 2010, Lampadariou & Danovaro, 2010; Calvo et al. 2015), with the higher allelic richness found in central populations located in correspondence of the Siculo-Tunisian Strait. A first hypothesis able to explain the observed data was based on vicariance processes involving the differentiation of two main clades in Eastern and Western Mediterranean refugia. In this frame, the Strait was identified as a secondary contact zone where the two forms meet after the Last Glacial Maximum (Arnaud-Haond et al. 2007; Serra et al. 2010). This pattern would be maintained by the present water circulation regime (Serra et al., 2010). A more recent study (Chefaoui et al. 2017) confirmed the relevance of Siculo-Tunisian Strait, where the authors found the highest

allelic richness, according to previous studies. These Authors used an Ecological Niche Modeling and compared their results with the genetic population structuring of *P. oceanica*. This allowed producing new evidence suggesting that the central Mediterranean, and in particular the Siculo-Tunisian Strait area, could be the main glacial refuge during the Last Glacial Maximum. This would imply that the higher genetic diversity shown by resident seagrass populations would be due to long term persistence. As *P. oceanica* is a foundational seagrass, the existence of this refugial area could have also contributed to the maintenance of associated biota during glacial period.

Nowadays *P. oceanica* is declining especially in the western basin, despite this is the area with the higher probability of occurrence and with the highest diversity in terms of associated species richness (Chefaoui et al., 2017). The fragmentation and the disappearance of the meadows, could threaten the survival of key populations and much of the species associated (Telesca et al., 2015) which are important components of this biodiversity rich ecosystem.

1.3 The target taxon: Amphipoda

Amphipods are the most numerically important order among Peracarid crustaceans, with more than 10.000 described species. The traditional higher classification of Amphipoda order was based only on morphological characters, and families were traditionally subdivided in alphabetical order (Barnard & Karaman 1991, Martin & Davis 2001). But nowadays a new classification based on phylogeny is proposed by Lowry & Myers, In 2017 the authors recognised six suborders: Amphilochidea; Colomastigidea; Hyperiidea; Hyperiopsidea; Pseudoingolfiellidea and Senticaudata. This new classification exceeds the traditional one, not based on phylogenetic, which subdivided amphipods in four main groups.

Amphipods exhibit a large variability in morphology and it is not easy to describe a common habitus shared by all the species of this taxon. However, some morphological features able to define a general ‘type amphipod’ do exist. A common amphipod is a small crustacean with a latero compressed body divided into three parts: the head or cephalotorax, the thoracic part or pereon and the abdomen or pleon. Amphipods are dioecious and most species present a remarkable sexual dimorphism (Conlan, 1991). The most evident common feature is the presence of a marsupium in

females, that hosts the eggs. Mating behaviour varies but in general a male finds a mature female using antennae, then with gnathopods grabs the female during the mating. Fertilization occurs in the marsupium, where the eggs are ventilated by pleopods while developing (Dick, 1998). From the eggs subsequent larval stages develop within the marsupium and when, after some weeks, juvenile amphipods reach a morphology similar to the adult form they are released into the environment (Karaman, 1991). Due to this peculiar direct development of larvae, amphipods totally lack a pelagic larval phase and their dispersal is therefore quite limited and mainly due to buoyancy associated to drift objects. Amphipod species inhabiting Mediterranean Sea exhibit overall a fast development and growth and a relatively short life-cycle, in the range of 4-6 months up to 2 years (Bellan-Santini 1998, 1999; Delgado et al., 2009).

Amphipods inhabit a variety of environments from terrestrial to freshwater areas, including interstitial to underground waters, coastal and pelagic marine waters, deep sea and abyssal trenches. They are able to establish various symbiotic associations with ascidians or planktonic animals and some species are also ectoparasites of marine vertebrates. These heterogenous habits may be at least in part explained by the high diversification of their trophic plasticity, recorded in all the ecosystems where they live: amphipods are detritivores, suspension or deposit feeder, parasites, carnivorous, predators etc. (Bellan-Santini, 1999).

Their wide distribution and the high richness of species put the amphipods in a position to be used in the field of ecological indicators since they are particularly sensitive to the presence of polluted sediments and to dredging, showing an overall decline in the abundance and diversity of their assemblages (Gómez Gesteira & Dauvin, 2000; Dauvin & Ruellet, 2007, 2009). For this reason they are used as reliable bioindicators in marine coastal environments.

As mentioned early, *P. oceanica* meadows are characterized by high levels of biodiversity, with the vagile fauna as a fundamental component in this ecosystem (Buia et al., 2000). In reference to *P. oceanica*, the term vagile fauna indicates all the organisms associated to leaves, rhizomes and litter, free to move independently. The main taxonomic groups are molluscs, polychaets, annelids, echinoderms and crustaceans. Among them, amphipods represent one of the most important and abundant taxonomic groups (Mazzella et al., 1989; Gambi et al., 1992; Sturaro et al., 2015). The meadow is more than a trophic resource for amphipods, which may actively choose the seagrass as a substratum to find protection from predation or to complete their life cycle (Sanchez-Perez, 1999).

Here they represent an important food source for higher level predators such as fish (Bell & Harmelin-Vivien, 1983; Pinnegar & Polunin, 2000). In *P. oceanica*, amphipods community can be defined as a complex assemblage of species, and in the meadow they reach high numerical abundance and specific diversity compared to un-vegetated bottoms (Scipione & Zupo, 2010). However, totally exclusive taxa living in this biotope only do not exist (Buia et al., 2000). A great spatial variability of amphipod assemblages has been recorded in the meadows of Mediterranean Sea and even though some common features exist, amphipods communities are very variable in different areas.

1.4 Main Objectives

The general objective of this study is to analyse the mechanisms involved in generating diversity patterns in the marine coastal environment, using *P. oceanica* and associated amphipods as study system. Although amphipods are often regarded as key-components of *P. oceanica* meadows ecosystem for their important role in energy transfer through the food web, relevant knowledge is still poor on many subjects, from taxonomy to ecology, from community structure to phylogeography.

To deepen the knowledge on amphipod diversity we decided to investigate their biogeographic patterns approaching different levels of biodiversity: from community to molecular. We have therefore used different kinds of data (species abundance, incidence data mined from literature, sequence data from mitochondrial and nuclear genes) and approaches (classical community statistics, network and modularity, phylogeography) to provide a first picture of the mechanisms underlying the diversity pattern of amphipods associated to *P. oceanica*. According to this scheme, the research is subdivided in three parts:

- 1) We have investigated the temporal variation of amphipod assemblages using classical statistical approaches to community structure. The variation in assemblages' composition was studied during a year-round survey in the Tyrrhenian Sea, by sampling two localities in Giannutri Island (Tuscan Archipelago). Diversity and multivariate analyses were used to evaluate the role of spatial vs. temporal factors in structuring amphipod assemblages in the aim of evaluating if the well known life cycle of the plant during the whole year influenced the diversity and composition of local communities. The results of this study have been published on *Crustaceana* in a paper entitled "Seasonal variations of amphipod assemblages in a *Posidonia oceanica* meadow from central

Tyrrhenian Sea (Italy)”, which is presented as Chapter 2 of this thesis.

- 2) We studied the diversity patterns of *P. oceanica* amphipods across the Mediterranean Sea by applying a network approach using incidence data mined from literature. The aim in using this approach was to infer biogeographic modules and the biogeographic roles of associated localities too. The specific objectives of this research were: 1) to detect the biogeographic subdivision of amphipod assemblages across the whole Mediterranean Sea (i.e. biogeographic modules); 2) to check if amphipods diversity may reflect the generalised biogeographic patterns recorded for other groups across the basin, and if the most relevant geographic barriers influenced amphipods distribution; 3) to check if the identified biogeographic modules differed not only in their species richness and locality role, but also in the biogeographic distribution and range width of the species found in the various assemblages; 4) to explore the relationships between assemblage diversity and the paleogeographic history of *P. oceanica*. Both network analysis and modularity represent an innovative methodology to investigate the biogeographical role of localities and this research is the first attempt to use such approach to shed light on the biogeography of Mediterranean benthic communities. The results of this study are now in publication on *PeerJ* in a paper entitled “Biogeography of Mediterranean amphipods associated with *Posidonia oceanica* meadows”, which is presented as Chapter 3 of this thesis.
- 3) We have carried out a phylogeographic analysis of two species belonging to the genus *Apolochus*, which is one of the most common and widely distributed amphipods in *P. oceanica* meadows. We used both mitochondrial (COI) and nuclear (Histone 3) markers in the aim of: 1) to review the present taxonomic arrangement and to detect possible hidden or cryptic species; 2) to study the effects of the main geographic barriers and of the paleoclimatic history of the Mediterranean Sea on the genetic structure of the species investigated; 3) to verify if deep splits are engraved in the genetic diversity of these species, since there is growing evidence that species with low dispersal ability (as benthic amphipods) retain the signature of ancient events which are on the contrary erased by the high contemporary gene flow characterizing highly vagile species. The results of this study are now going to be submitted for publication in a paper entitled “Cryptic diversity and ancient divergence in two species of Amphipods from central Mediterranean Sea”, which is presented as Chapter 4 of this thesis.

2. SEASONAL VARIATIONS OF AMPHIPOD ASSEMBLAGES IN A *POSIDONIA OCEANICA* (LINNAEUS) DELILE, 1813 MEADOW FROM CENTRAL TYRRHENIAN SEA (ITALY)

FEDERICA CAMISA, CHIARA ABBATTISTA, BRUNO BELLISARIO, DARIO ANGELETTI, LORETTA LATTANZI & ROBERTA CIMMARUTA

ABSTRACT

Temporal variability in species composition and abundance may enhance coexistence in ecological communities, especially when subjected to strong seasonal patterns. Species distribution through time may be related to many factors, such as changes in the structure and functioning of preferred habitats, variation in ecological interactions or asynchronous patterns in species life cycles. Here, data from one-year survey of amphipods assemblages from a nearly undisturbed *Posidonia oceanica* meadow are used to investigate the seasonal patterns in species abundance and identity. Our results highlight the presence of a core group of species inhabiting the meadow throughout the year, although an in depth analysis reveals three differentiated seasonal assemblages corresponding to autumn, winter, and a warm season clustering of both spring and summer. The observed patterns, compared to the knowledge about seagrass seasonality and amphipod ecology, suggest the interplay between different mechanisms drive the coexistence of amphipods over the whole year, including niche partitioning, predation pressure, and variations in life history traits

Key words - *Posidonia oceanica*, amphipods, seasons, niche, ecological interactions, Tuscan Archipelago

RIASSUNTO

Variazioni temporali nella composizione e abbondanza delle specie sono alla base della coesistenza interspecifica nelle comunità ecologiche, specialmente quando si registra una forte stagionalità. La distribuzione temporale delle specie può essere correlata a molti fattori, quali i cambiamenti nella struttura e nel funzionamento degli habitat preferiti, le variazioni nelle interazioni ecologiche oppure l'asincronia dei cicli vitali delle specie. In questo lavoro, sono state studiate per un anno le comunità di anfipodi di una prateria di *Posidonia oceanica* scarsamente interessata da disturbo antropico, al fine di indagare i modelli stagionali di abbondanza e distribuzione delle specie. I risultati evidenziano la presenza di un gruppo di specie che abita la prateria durante tutto l'anno, anche se un'analisi più approfondita evidenzia profondi differenziamenti della struttura di comunità corrispondenti all'autunno, all'inverno e ad una stagione calda costituita da primavera ed estate. I pattern osservati, se confrontati con le pregresse conoscenze della variazione stagionale dei posidonieti e dell'ecologia degli Anfipodi, suggeriscono che sia l'interazione tra più meccanismi a promuovere la coesistenza tra le varie specie durante l'anno quali ad esempio la suddivisione della nicchia, la pressione di predazione e le variazioni nelle caratteristiche dei cicli vitali.

Parole chiave: *Posidonia oceanica*, Anfipodi, stagioni, Nicchia, Interazioni ecologiche, Arcipelago Toscano

2.1 INTRODUCTION

Environmental temporal variations are thought to play a relevant role in both defining species abundance within communities and promoting species coexistence, with direct and indirect mechanisms involved in controlling the diversity and composition of ecological communities across time (Shimadzu et al., 2013). Niche differentiation, along with the spatial and temporal partitioning of resources (see McMeans et al., 2015 and references herein), allows for species-specific responses to both types of environmental variations and limiting factors are listed among the most relevant of these mechanisms (Chesson, 2000; Ruokolainen & Hanski, 2016). Seasonal fluctuations are evident, and marked temporal variations are known to influence species abundance, although the consequences of this variation for species coexistence are still under investigation. These studies are

especially needed in the marine environment, where the seasonal patterns may differ from those recorded on land (Shimadzu et al., 2013). The seasonal changes of marine habitats may influence key life-history features of different species, such as developmental and growth rates, species migration, spawning or nesting time, and even offspring sensitivity to acidification (e.g. Murray et al., 2014; Urrea et al., 2013).

In the Mediterranean Sea, seagrass meadows are one of the most complex and important ecosystems, and are subjected to strong seasonal fluctuations in plants' biological features, such as biomass and productivity (Guidetti et al., 2002). *Posidonia oceanica* (Linnaeus) Delile, is the dominant seagrass and is one of the most important primary producers in coastal waters by playing a fundamental ecological role as a habitat-forming species (Kendrick et al., 2005). The three-dimensional structure of the meadows, consisting of a thick foliar canopy and of an articulate layer of roots and rhizomes, helps develop complex epiphyte assemblages and offers a variety of suitable microhabitats hosting hundreds of animals and plant species, as well as fungi and micro-organisms (Buia et al., 2000; Borg et al., 2006; Bedini et al., 2011; Mascart et al., 2015). Among invertebrates, amphipods are one of the most abundant and diverse groups of the vagile fauna associated with *P. oceanica* (Mazzella et al., 1989; Gambi et al., 1992; Sturaro et al., 2015). These small peracaridean crustaceans without pelagic larval stages, are known to maintain a close relationship with the substrate and are sensitive to environmental changes, so their community composition is widely used as an environmental indicator (de-la-Ossa-Carretero et al., 2012; Marusso et al., 2013). Within *P. oceanica*, amphipods play an important role by transferring energy from lower to higher trophic levels (Michel et al., 2015; Zaabar et al., 2015). Furthermore, *P. oceanica* is more than a trophic resource for amphipods, which may actively choose seagrass as a substratum to find protection from predation or to complete their life cycle (Sanchez-Jerez, 1999; Sturaro et al., 2016).

Given the complexity of understanding temporal patterns and relationships between *P. oceanica* and amphipods, as well as the need to improve community-based analyses, a yearly survey of amphipod assemblage structure from a little impacted *P. oceanica* meadow located in Giannutri Island (central Tyrrhenian Sea) was carried out. The aim of this study is to investigate temporal changes in the structure of amphipod assemblages across the seasons.

2.2 MATERIALS AND METHODS

Study area

Investigations were carried out in a *Posidonia oceanica* meadow from Giannutri Island (central Tyrrhenian Sea, Italy). This small limestone island, characterized by a rugged and rocky coastline, is part of the National Park of Tuscan Archipelago and a Special Area of Conservation (Habitats Directive 92/43/EEC, SACIT51A0024). The seagrass meadows around the island cover about 32 ha and are characterized by a coastal extension with a sharp lower limit due to the slope of the rocky seabed and the low amount of shallow bays (Borriello et al., 2010). Samples were taken from two sites, Punta Secca (A) and Secca di Punta Secca (B) (fig. within Zone 2 of the protected area, which is nearly undisturbed since human activities need to be authorized by the Park authority.

Sampling

Samples were collected by SCUBA diving at a constant depth between 15-20 m within 40x40 cm quadrats (0.16m²) using an air-lift sampler (500-µm mesh size), as described in Michel et al. (2010). The suction time was the same for each sample collected (3 min). At each site, three replicates at intervals of 10 m range were taken in February, May, August and November 2014, for a total of six samples per season. Samples were preserved in 70% ethanol until sorted out in the lab using a stereomicroscope. Identification to the species level was based on the handbooks by Ruffo (1982, 1989, 1993, 1998), the nomenclature was updated according to the World Register of Marine Species (WoRMS, last access January 2017).

Statistical analyses

The structure of amphipod assemblages between seasons and sites was compared by a two-way permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001), with Season (*Se*, four levels) and Site (*Si*, two levels) as fixed factors and Replicate (*Re*, three levels) as a random factor nested in Site with 9.999 permutations. A non-metric multidimensional scaling analysis (nMDS), based on abundance data, was performed to visualize multivariate patterns. The goodness

of fit statistics in the nMDS ordination diagram was assessed using the Kruskal stress value (Kruskal & Wish, 1978), and considering 0.12 as the threshold value for a reliable ordination (Clarke, 1993). PERMANOVA and nMDS analyses were based on the Bray-Curtis similarity matrices calculated from the double square root transformation of abundance data, to maintain the contribution of rarest species without over-weighting. This bulk of analyses allowed for defining three temporally homogeneous clusters of seasons, which were then used to perform any further analysis. A similarity percentage analysis (SIMPER, Clarke & Warwick, 2001) was performed to identify the species that mostly contributed to dissimilarity among temporal clusters. The temporal distribution of these species was highlighted by means of a ternary plot, which graphically depicts the proportion of species abundance in the identified clusters and has the advantage of plotting three-variable data in a two-dimensional plot. The overall number of species (S), the Shannon-Wiener diversity index (H'), and the dominance based on the Simpson diversity index (D) were calculated from mean seasonal values, and potential differences were estimated by a bootstrap t -test (Magurran, 2004). Statistical analyses were performed using PAST 3.12 (Hammer et al., 2001) and PRIMER v6 (Clarke & Gorley, 2006).

2.3 RESULTS

A total of 873 individuals were sampled, and 718 were identified to the species level, belonging to 62 species and 27 families (table I). Overall, the most abundant species was *Apherusa chiereghinii* Giordani-Soika, 1949, with 110 individuals, followed by *Liljeborgia dellavallei* Stebbing, 1906 (105 specimens) and *Apolochus neapolitanus* (Della Valle, 1893) (91 specimens).

The two-way PERMANOVA showed no significant influence of the factor Si ($F = 1.641$; $p = 0.078$) on the structure of amphipod assemblages. This showed spatial homogeneity among study sites, so all samples (Punta Secca and Secca di Punta Secca) can be considered as part of a unique locality. The two-way PERMANOVA also highlighted a significant influence of factor Se ($F=2.965$; $p < 0.001$). Yet no differences in amphipod assemblages between spring and summer were identified (pairwise comparison, $F = 1.013$; $p = 0.104$), which can therefore be considered as a single temporal group. Winter and autumn assemblages differed among themselves ($p < 0.05$) and from the above

mentioned spring- summer season ($p < 0.05$ in all cases). These differences were highlighted by the pattern of ordination in the nMDS plot, which showed a clear-cut separation of the autumn from all other seasons (fig. 2). Autumn samples laid separately from all the others and exhibited a higher dispersion, while the winter samples were closer to the spring-summer ones.

This pattern was confirmed by the SIMPER analysis that showed a relatively low dissimilarity in the assemblage of amphipods between winter and spring-summer (average dissimilarity = 49.25) with 14 species responsible for about 50% of the differentiation (table II). Higher differences were found comparing autumn versus both spring-summer and winter (average dissimilarity 67.79 and 65.29, respectively), thus confirming the high peculiarity of the assemblage in this season. The temporal distribution of the species responsible for most of the differentiation between the three time periods showed two dominant species, *Gammarella fucicola* (Leach, 1814) and *Socarnes filicornis* (Heller, 1866), strictly related to autumn samples, while *Leucothoe euryonyx* (Walker, 1901) and *Deflexilodes subnudus* (Norman, 1889) were fairly related to winter samples (fig. 3). *Cressa mediterranea* Ruffo, 1979, *Orchomene humilis* (A. Costa, 1853), *Iphimedia minuta* G. O. Sars, 1883, *Gitana sarsi* Boeck, 1871 and *Phtisica marina* Slabber, 1769 were instead associated with spring-summer time. It is worth noting that the most abundant species recorded: *A. chiereghinii*, *L. dellavallei* and *A. neapolitanus* were not strictly associated with any season (Figure 3).

Assemblage parameters, calculated from mean seasonal values, showed significant variations among all temporal clusters (Figure 4), with significant differences in terms of the Shannon diversity index and number of species (t -test, $p < 0.05$ in all cases). Higher values of dominance were found in autumn compared with spring-summer and winter assemblages (t -test, $p < 0.05$ in both cases), which did not show any significant difference among each other (t -test, $p = 0.71$). (Figure 4)

2.4 DISCUSSION

The results showed the presence of a pool of species (*A. chiereghinii*, *A. neapolitanus*, *L. dellavallei*, *Dexamine spinosa* (Montagu, 1813) and *Apolochus picadurus* (J. L. Barnard, 1962) found throughout the whole year in the studied *P. oceanica* meadow, which is in agreement with other observations from the Mediterranean Sea (Zakhama-Sraieb et al., 2006, 2011; Scipione & Zupo,

2010; Bellisario et al., 2016). However, a more in depth analysis based on year-round data showed a strong seasonal variation in the species composition of amphipod assemblages, along with their abundance and diversity. This pattern identified three main seasonal groups, represented by autumn, winter, and the warm season (e.g. spring and summer). During the warm season, amphipod assemblages were characterized by a high number of individuals partitioned in a relatively high number of species, thus causing a significant contraction in terms of species dominance. During autumn, the structure of amphipod assemblages changed and became dominated by two species, *S. filicornis* and *G. fucicola*. Winter showed the lowest values in terms of species diversity and abundance, and two weakly associated species, *L. euryonyx* and *D. subnudus*. Similar seasonal patterns were already observed in Peracarids associated with other seagrasses like *Zostera* (*Zostera marina* Linnaeus, 1753 and explained by the plant seasonality in growth and production rates (Ott, 1980; Karaçuha et al., 2009; Esquete et al., 2011; Zaabar et al., 2015). The link between seasonal fluctuations and species coexistence has recently been shown in a long term study on an estuarine fish community, while studies on crustaceans are still lacking (Shimadzu et al., 2013).

The data presented here refers to a single year and do not concern seagrass meadow features, which prevents a direct comparison with changes in amphipod assemblages. However, some general considerations can be drawn from the seasonal patterns of *P. oceanica* life cycle, suggesting that amphipod assemblages and species coexistence may be promoted by different mechanisms, mostly related to direct and indirect effects of the changing host plant structure. A possible explanation for the high number of species observed during the warm season may involve a more efficient partitioning of the abundant food resources available at that time, implying a reduction in trophic niche overlap. Indeed, recent studies highlighted the existence of interspecific differences in the feeding habits of most amphipods, especially within the preferred grazing compartment of each species (e.g. *A. chiereghinii*, *Aora spinicornis* Afonso, 1976 or *G. aequicauda*) (Michel et al., 2015). During the warm season, seagrasses reach their highest productivity and show a peak in epiphyte assemblages, as well as in their spatial coverage (Ott, 1980; Bedini et al., 2011), allowing for selective foraging by amphipods, which are known to exhibit strong species-specific dietary preferences (Michel et al., 2015). This feeding behaviour may have a direct effect on amphipod coexistence through niche subdivision, but also an indirect one through a facilitation process on the epiphytic community with grazing activity increasing the diversity of epiphytes, thus promoting the

coexistence of even more amphipods (Duffy et al., 2001; Duffy et al., 2003; Jaschinski et al., 2008). However, the spatial partitioning of food resources is not the only mechanism able to explain the high diversity of amphipods in our samples, since a certain degree of temporal partitioning should be considered. Many amphipod species show a diel vertical migration behaviour, so they use the lower layers of the meadows during the day and migrate to the higher foliar stratum during the night for grazing activity (Sanchez Jerez et al., 1999; Michel et al., 2015). Moreover, the habitat complexity during the warm period would allow a reduction in the predation pressure by high-order predators, which has been pointed out as related to seasonal changes in the number and diversity of amphipod species (Nelson, 1979). As suggested by Sturaro et al. (2016), this can be relevant for amphipods which have been demonstrated to show species-specific responses to predation, which are probably related to their life history strategies and ecological behaviour. During the autumn, the structure of amphipod assemblages changed and became dominated by two species, *S. filicornis* and *G. fucicola*, which are mainly detritivorous with a degree of a mixed diet (Guerra-Garcia et al., 2014). The relatively high species diversity and the dominance of few species, however, suggest that other mechanisms, unrelated to trophic habits, may act to determine the observed patterns of amphipod assemblages in autumn. For example, increasing predation pressure may selectively involve more vulnerable species whose life traits and/or ecological behaviours can not counteract the progressive decrease of habitat complexity due to the decay of leaf tips (Ott, 1980; Gallmetzer et al., 2005). Accordingly, species, such as *G. fucicola*, have an advantage by being able to both feed on epiphytes from the whole plant, including leaves, litter, and rhizomes, and accomplish its whole biological cycle within the seagrass litter, which provides an effective refuge against predation (Lepoint, 2006; Michel et al., 2015). Along the seasonal succession, seagrass' productivity is least in winter, when young leaves start developing, which is associated with the lowest production of epiphytic biomass (Ott, 1980). The high hydrodynamic regime typical of this season and the sparse canopy provided by the short leaves both strongly affects the removal of leaf litter and the settlement of individuals (Hovel et al., 2002; Mascart et al., 2015), likely explaining the lower diversity of winter amphipod assemblages.

Although limited in space and time, our results reinforce the view that the relationship between *P. oceanica* and the associated amphipods should be highly complex (Sturaro et al., 2015), implying multiple scales and interactive factors which can mould local assemblages through the year. These

regulating factors include, but are not limited to, the interplay between spatial and temporal niche partitioning driven by the seasonal changes in the structure and functioning of the seagrass. Other mechanisms may equally contribute to assemblage composition through seasons, such as predation pressure or life history strategies, although their effects at the assemblage level are still unclear (Sturaro et al., 2014). Further studies expanding the spatial and temporal investigation of the seasonal patterns in assemblage composition, possibly coupled with seasonal seagrass feature variation, may shed light into the mechanisms behind the temporal variation of amphipod assemblages in *P. oceanica*

2.5 TABLES AND FIGURES

Table 1 - List of identified species.

Suborder	Family	Species	Winter	Spring	Summer	Autumn
Gammaridea	Ampeliscidae	<i>Ampelisca rubella</i> A. Costa, 1864		1		
		<i>Ampelisca tenuicornis</i> Liljeborg, 1855		2		
	Amphilochidae	<i>Apolochus neapolitanus</i> (Della Valle, 1893)				1
		<i>Apolochus picadurus</i> (J.L. Barnard, 1962)				1
	Atylidae	<i>Gitana sarsi</i> Boeck, 1871	15	39	23	33
		<i>Atylus veddomensis</i> (Bate & Westwood, 1862)	32	17	12	27
		<i>Nototropis guttatus</i> Costa, 1853	27	1	31	5
	Cressidae	<i>Cressa cristata</i> Myers, 1969		1		
		<i>Cressa mediterranea</i> Ruffo, 1979		7		
	Cyproideidae	<i>Peltocoxa gibbosa</i> (Schiecke, 1977)				1
		<i>Peltocoxa mediterranea</i> Schiecke, 1977		1	2	1
	Dexaminidae	<i>Dexamine spiniventris</i> (Costa, 1853)	4	1	2	
		<i>Dexamine spinosa</i> (Montagu, 1813)	7	2	1	
		<i>Guernea (Guernea) coalita</i> (Norman, 1868)		3	2	
	Iphimediidae	<i>Iphimedia minuta</i> G. O. Sars, 1882	2	15	19	9
		<i>Iphimedia vicina</i> Ruffo & Schiecke, 1979		2	1	1
	Leucothoidae	<i>Leucothoe euryonyx</i> (Walker, 1901)				1
		<i>Leucothoe serraticarpa</i> Della Valle, 1893		1		41
		<i>Leucothoe spinicarpa</i> (Abildgaard, 1789)	1			
		<i>Idunella nana</i> (Krapp-Schickel, 1975)	1			5
	Liljeborgidae	<i>Liljeborgia dellavallei</i> Stebbing, 1906	15	18	29	4
		<i>Lysianassa costae</i> (Milne-Edwards, 1830)			1	
	Lysianassidae	<i>Orchomene grimaldii</i> Chevreux, 1890				1
		<i>Orchomene humilis</i> (Costa, 1853)				1
		<i>Orchomene massiliensis</i> Ledoyer, 1977				1
		<i>Orchomene similis</i> Chevreux, 1912	1			
		<i>Socarnes filicornis</i> (Heller, 1866)				1
		<i>Tryphosa nana</i> (Krøyer, 1846)	1	9	7	1
		<i>Tryphosella minima</i> (Chevreux, 1911)		1		
		<i>Deflexilodes subnudus</i> (Norman, 1889)			1	
		<i>Periocolodes aequimanus</i> (Korssman, 1880)		2		
		<i>Pontocrates arenarius</i> (Bate, 1858)	4			1
	Opsidae	<i>Synchelidium longidigitatum</i> Ruffo, 1947	1			
		<i>Normanion chevreuxi</i> Diviacco & Vader, 1988	2	3	1	
	Phoxocephalidae	<i>Harpinia antennaria</i> Meinert, 1890	38	14	39	14
		<i>Harpinia crenulata</i> (Boeck, 1871)	1	5	1	4
		<i>Harpinia zavodniki</i> Karaman, 1987				3
		<i>Metaphoxus simplex</i> (Bate, 1857)				8
	Stenothoidae	<i>Stenothoe dollfusi</i> Chevreux, 1887				2

	Uristidae	<i>Ichnopus taurus</i> Costa, 1853	3			
		<i>Tmetonyx nardonis</i> (Heller, 1866)	2		1	
Senticaudata	Aoridae	<i>Aora gracilis</i> (Bate, 1857)			2	1
		<i>Aora spinicornis</i> Afonso, 1976	4			
		<i>Microdeutopus armatus</i> Chevreux, 1886			3	2
	Calliopiidae	<i>Apherusa chiereghinii</i> Giordani-Soika, 1949	1			2
	Caprellidae	<i>Caprella acanthifera</i> Leach, 1814	3	6	14	1
		<i>Phtisica marina</i> Slabber, 1769		3		
	Cheirocratidae	<i>Cheirocratus sundevallii</i> (Rathke, 1843)			1	
	Corophiidae	<i>Leptocheirus guttatus</i> (Grube, 1864)		9	1	1
		<i>Leptocheirus pilosus</i> Zaddach, 1844			1	
		<i>Erichthonius punctatus</i> (Bate, 1857)		2	3	
	Ischyroceridae	<i>Siphonoecetes (Centraloecetes) dellavallei</i> Stebbing, 1899		1	1	1
		<i>Siphonoecetes (Centraloecetes) neapolitanus</i> Schiecke, 1979		6	3	1
		<i>Microjassa cumbrensis</i> (Stebbing & Robertson, 1891)	5	11	7	1
	Kuriidae	<i>Micropythia carinata</i> (Bate, 1862)			2	
	Maeridae	<i>Maera grossimana</i> (Montagu, 1808)		6	3	
		<i>Hamimaera hamigera</i> (Haswell, 1879)	2	9		3
	Nuuanuidae	<i>Gammarella fucicola</i> (Leach, 1814)				7
	Phliantidae	<i>Pereionotus testudo</i> (Montagu, 1808)		1	1	
	Photidae	<i>Gammaropsis dentata</i> Chevreux, 1900	12	7	14	2
		<i>Gammaropsis maculata</i> (Johnston, 1828)			1	
	Pontogeneiidae	<i>Eusiroides dellavallei</i> Chevreux, 1899	1			

Table 2 - SIMPER analysis showing dissimilarities in amphipod composition (expressed in percentage) between the three seasonal groups (W = Winter; Sp-S = Spring-Summer; A = Autumn) and, the average abundances, the contribution (σ_i), relative contribution (σ_i/SD) of most important species.

Dissimilarity	Species	Average abundances		σ_i	σ_i/SD
		W	Sp-Sn		
49.25	<i>Deflexilodes subnudus</i>	0.74	0.25	4.43	1.20
	<i>Iphimedia minuta</i>	0.17	0.70	4.40	1.14
	<i>Phtisica marina</i>	0.56	0.87	4.30	1.10
	<i>Apolocus neapolitanus</i>	1.48	1.01	4.11	1.03
	<i>Deixamine spinosa</i>	1.10	1.07	4.04	1.08
	<i>Orchomene humilis</i>	0.22	0.57	3.92	0.90
	<i>Apolocus picadurus</i>	1.37	1.05	3.87	1.19
	<i>Cressa mediterranea</i>	0.39	0.36	3.73	0.87
	<i>Synchelidium longidigitatum</i>	1.13	0.79	3.70	1.03
	<i>Leucothoe euryonyx</i>	0.53	0.00	3.51	0.95
	<i>Liljeborgia dellavallei</i>	1.52	1.23	3.47	0.92
	<i>Gitana sarsi</i>	1.05	1.36	3.31	0.90
	<i>Siphonoecetes neapolitanus</i>	0.20	0.40	3.09	0.79
	<i>Apherusa chiereghinii</i>	1.22	1.35	2.90	1.17
67.71		Sp-Sn	An		
	<i>Gammarella fucicola</i>	0.08	1.38	6.72	1.90
	<i>Gitana sarsi</i>	1.36	0.40	5.11	1.55
	<i>Apolocus picadurus</i>	1.05	0.25	5.06	1.46
	<i>Phtisica marina</i>	0.87	0.17	4.08	1.38
	<i>Apolocus neapolitanus</i>	1.01	1	3.95	1.20
	<i>Liljeborgia dellavallei</i>	1.23	0.87	3.65	1.18
	<i>Perioculodes aequimanus</i>	0.40	0.80	3.58	1.21
	<i>Synchelidium longidigitatum</i>	0.79	0.33	3.39	1.21
	<i>Iphimedia minuta</i>	0.70	0.17	3.26	1.14
	<i>Apherusa chiereghinii</i>	1.35	1.27	3.16	1.02
	<i>Socarnes filicornis</i>	0.00	0.58	3.05	0.91
	<i>Orchomene humilis</i>	0.57	0.17	2.84	0.92
	<i>Deixamine spinosa</i>	1.07	0.95	2.68	0.96
65.29		An	Wn		
	<i>Gammarella fucicola</i>	1.38	0.00	7.82	2.04

<i>Apolocus picadurus</i>	0.25	1.37	7.00	2.48
<i>Gitana sarsi</i>	0.40	1.05	4.76	1.44
<i>Synchelidium longidigitatum</i>	0.33	1.13	4.57	1.52
<i>Perioculodes aequimanus</i>	0.80	0.00	4.39	1.33
<i>Apolocus neapolitanus</i>	1.00	1.48	4.34	1.14
<i>Liljeborgia dellavallei</i>	0.87	1.52	4.31	1.22
<i>Deflexilodes subnudus</i>	0.01	0.74	4.24	1.35
<i>Socarnes filicornis</i>	0.58	0.00	3.45	0.91
<i>Phtisica marina</i>	0.17	0.56	3.36	0.99
<i>Apherusa chiereghinii</i>	1.27	1.22	3.27	1.26

Figure 1 - Geographic location of the sampling area and sampling sites (A and B) in Giannutri Island (Italy). At each site, three replicates were sampled in each season Winter (W), Spring (Sp), Summer (S) and Autumn (A).

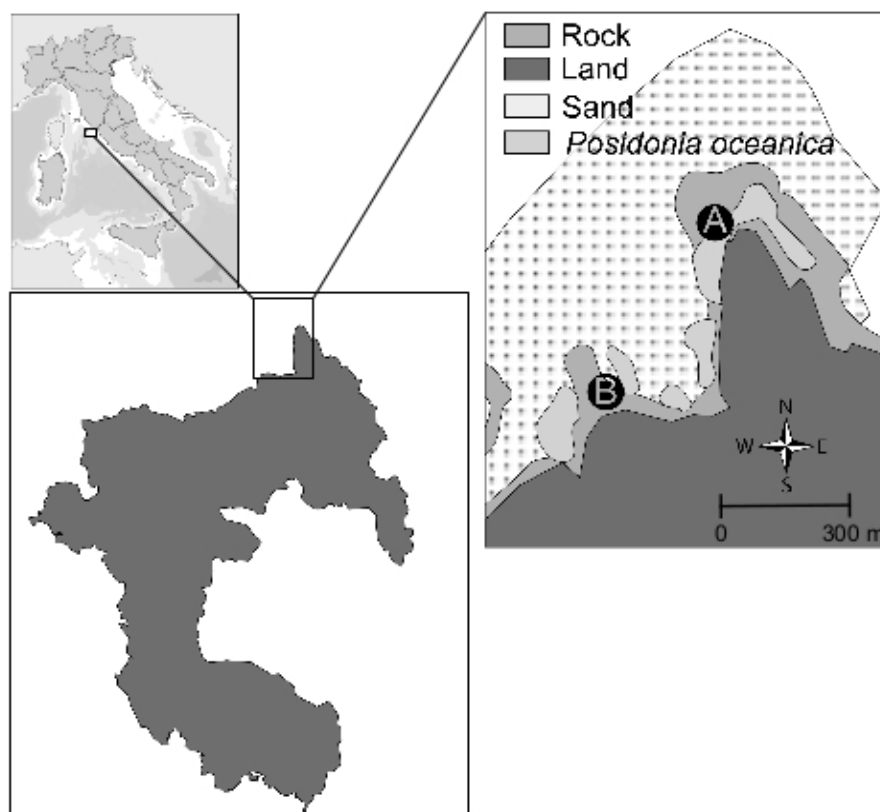


Figure 2 - Non-metric multidimensional scaling ordination (nMDS) based on the Bray-Curtis distance measured on abundance data.

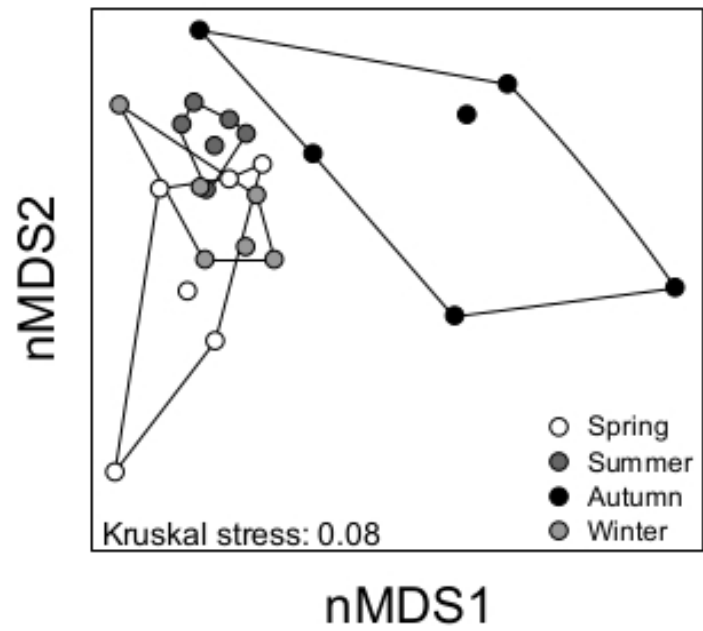


Figure 3 - Ternary plot showing the distribution of the main species, as identified by the SIMPER analysis, within the three seasonal groups (W = Winter; Sp-S = Spring-Summer; A = Autumn).

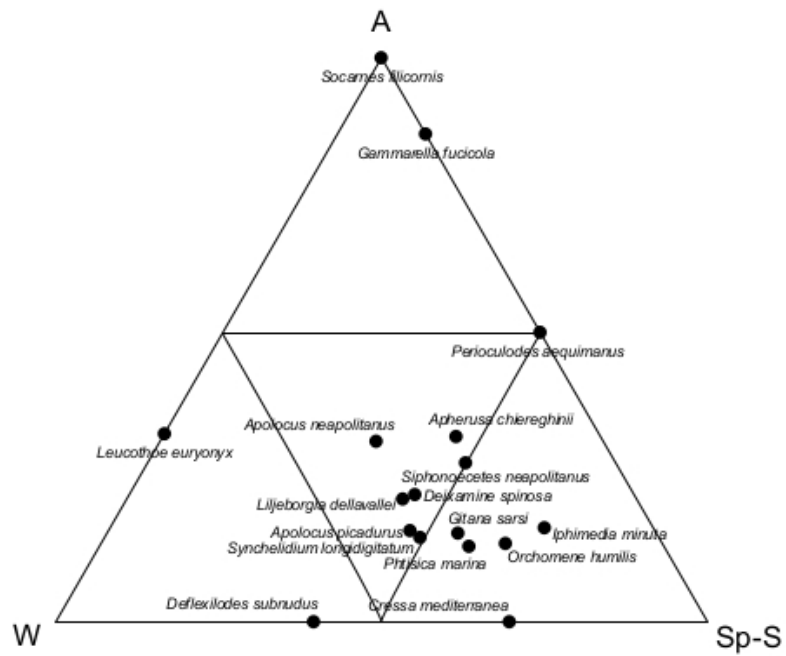
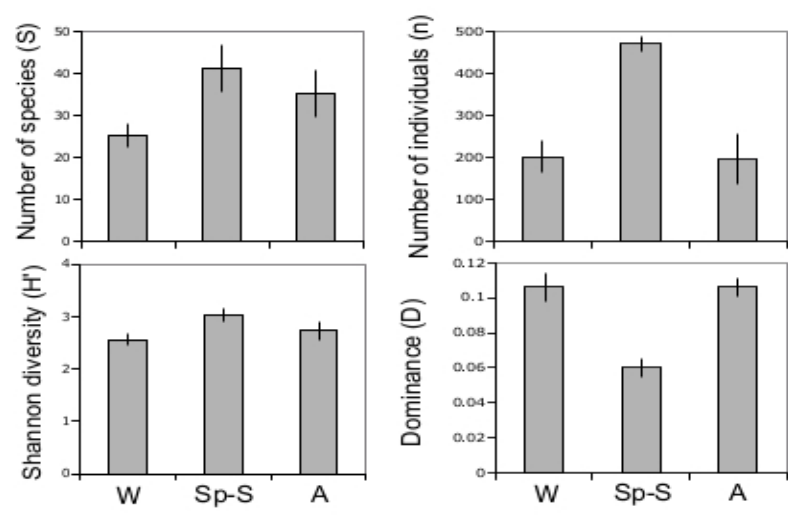


Figure 4 - Species number (S), Shannon diversity index (H'), number of individuals and dominance measured in the identified seasonal groups (W = Winter; Sp-S = Spring-Summer; A = Autumn). Values are given as mean \pm SD after 9,999 replicationss under a bootstrap t -test.



3. A NETWORK APPROACH TO IDENTIFY BIOREGIONS IN THE DISTRIBUTION OF MEDITERRANEAN AMPHIPODS ASSOCIATED WITH *POSIDONIA OCEANICA* MEADOWS.

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ABSTRACT

Although amphipods are key components of the macro-fauna associated with *Posidonia oceanica* meadows, to date no studies focused on the structure and diversity of their assemblages across the whole Mediterranean Sea. Here, we applied a network approach based on modularity on a dataset mined from literature to identify biogeographic modules and to assess the biogeographic roles of associated localities. We also correlated the patterns evidenced with the biogeographic distribution of amphipod groups by means of a multivariate analysis. Modularity analysis highlighted four biogeographic modules bounded by the main Mediterranean biogeographic divides and evidenced a decrease in species diversity along a NW-SE gradient. Assemblages associated with Central-Western Mediterranean and, to a lesser extent, Tunisian modules showed the highest species richness and were identified as hubs, characterized by species with regional distributions that behave as source in a biogeographic context. The paleogeographic history of the host seagrass and the ecology of associated amphipods, both suggest the joint effect of species persistence and post-Last Glacial Maximum expansion in explaining the pattern of amphipod distribution in the Mediterranean Sea.

3.1 INTRODUCTION

The Mediterranean Sea represents only 0.3% of ocean waters, although is a recognized hot-spot of biodiversity hosting about 17,000 species, which are between 4 to 18% of the global macrofauna (Coll et al., 2010; Bianchi et al., 2012). This high diversity stems from a combination of oceanographic, ecological and biogeographic features allowing the coexistence of species of Atlantic origin with temperate and subtropical organisms (Coll et al., 2010). Latitudinal clines of environmental variables (mainly temperature and salinity) combined with marine currents resulted in a generalised latitudinal gradient of both primary production and species richness, decreasing from north-eastern to south-western regions (Coll et al., 2010; Lejeusne et al., 2010).

All these ecological and biological features allowed identifying biogeographic sectors within the Mediterranean Sea, each characterized by both different biota and ecological parameters (Bianchi et al., 2012). Although general patterns of biodiversity distribution within the Mediterranean Sea have been well described (Coll et al., 2010; Bianchi et al., 2012), studies concerning patterns and mechanisms of species co-occurrence across the whole Mediterranean basin are restricted to a relatively small number of organisms (Arvanitidis et al., 2002; Gerovasileiou & Voultsiadou, 2012). Moreover, geographically widespread and ecologically broadly adapted groups have been only seldom studied according to habitat-related subdivisions (Sevastou et al., 2013).

In this work, we focused on crustacean amphipods associated with *Posidonia oceanica* (L.) Delile meadows, since they represent one of the most relevant components of the vagile fauna of this key seagrass, endemic of the Mediterranean Sea. *P. oceanica* plays a fundamental role as ecosystem engineering along the Mediterranean coasts. Its meadows provide important ecosystem functions, including oxygen production, food supplying and reduction of coastal erosion (Boudouresque, Mayot & Pergent, 2006). The complexity of this multi-layered and three-dimensional habitat allows a great variety of associated fauna to live into the canopy, rhizomes and mattes, making the meadows a strikingly biodiversity-rich habitat within the Mediterranean (Buia, Gambi & Zupo, 2000). Among the vagile fauna associated with meadows, crustacean amphipods are one of the dominant groups, showing high abundance and diversity of species (Mazzella, Scipione & Buia, 1989; Gambi et al., 1992; Sturaro et al., 2015). Amphipods are key ecological components of seagrass habitats, due to their role in transferring energy across the system, and represent an important trophic resource for higher predators such as fish (Pinnegar & Polunin, 2000; Zakhama-Sraieb, Ramzi-Sghaier & Charfi-

Cheikhrouha, 2011; Michel et al., 2015; Bellisario et al., 2016). They are considered mesograzers herbivores feeding on epiphytes (diatoms and macroalgae), on multiple types of algae and on associated detritus (Michel et al., 2015), establishing a sort of facilitative interaction with the host plant, by promoting the seagrass growth and obtaining protection against predation (Valentine & Duffy, 2006).

Despite the importance of amphipods in seagrass systems, a comprehensive study on their biogeographic patterns of organization focused on *P. oceanica* meadows at whole Mediterranean scale is still lacking. Available data concern mainly check-lists and local studies based on classical diversity index (Gambi et al., 1992; Diviacco, 1998; Como et al., 2008; Scipione & Zupo, 2010; Bedini et al., 2011; Zakhama-Sraieb, Ramzi-Sghaier & Charfi-Cheikhrouha, 2011; Sturaro et al., 2015), which can however foster biogeographic studies using innovative approaches. Recently, specific metrics rooted in network analysis have been successfully applied in a biogeographic context, outperforming classic approaches as clustering methods in the identification of bioregions (Carstensen & Olesen, 2009; Vilhena & Antonelli, 2015; Bloomfield, Knerr & Encinas-Viso, 2018). In particular, modularity (i.e., the tendency of a network to subdivide in densely connected modules or clusters) has proved powerful in detecting groups of areas and/or species closely connected together (i.e., biogeographical modules, *sensu* Carstensen et al., 2012; 2013). This approach also provides relevant insights into the processes driving the assembly of communities by evaluating the importance of each local assemblage (represented by nodes) in terms of network connectivity (Bloomfield, Knerr & Encinas-Viso, 2018). Specific metrics related to the number of links within and between biogeographic modules can be used as indirect estimators of richness and endemism and provide information on the source/sink role of localities (Carstensen et al., 2012; 2013; Bloomfield, Knerr & Encinas-Viso, 2018). In this work, we mined data from literature on the distribution of amphipods associated with *P. oceanica* meadows along the Mediterranean basin. We then used a network approach based on modularity: 1) to detect a biogeographic structure; 2) to correlate the patterns evidenced with the biogeographic distribution and ecological features of amphipod groups; 3) to compare amphipods diversity with the paleogeographic history of Mediterranean Sea and biogeographic history *P. oceanica* seagrass. The results obtained are discussed in the aim to provide insights on how amphipod diversity could have spread within the Mediterranean basin.

3.2 MATERIALS AND METHODS

Study area and starting dataset

An extensive survey of the literature was conducted in order to obtain all available information on the presence of amphipods from *P. oceanica* meadows across different regions of the Mediterranean Sea (a list of data sources is reported in Supplemental Materials & Methods S1 and reference therein). Data were selected according to sampling strategies and checked for possible taxonomic issues following the criteria explained in Supplemental Materials & Methods S1. The final dataset included 152 amphipod species from 28 localities: 9 located in Tunisia, 16 in Italy (Tyrrhenian, Adriatic and Ionian Sea), 2 in Spain and 1 in Corsica (France) (Supplemental Materials & Methods S1). Available data therefore covered a large portion of the Mediterranean basin (Fig. 1), and were distributed in regions characterized by different geographic, hydrological and geological features, as well as by differences in the potential connectivity due to the general circulation models (Bianchi & Morri, 2000; Bianchi, 2007; Berline et al., 2014).

Information about the biogeographic distribution of observed amphipods were obtained from Bellan-Santini & Ruffo (2003), which classified the over 400 species of Mediterranean benthic amphipods in twelve macro-categories on the basis of their current distribution: WM, West Mediterranean; EM, East Mediterranean; Adr, Adriatic Sea; ME, Mediterranean endemics; Afr, African coasts from Ceuta to Cap Vert; Ib, Iberian coasts; Fr, French coasts; Br, British coasts; Norw, Norwegian coasts; Arct, Arctic Sea; Ind-P, Indo-Pacific Ocean; Cosm, Cosmopolitan. Here, species with an Atlantic distribution were clumped in two main categories from the five proposed by Bellan-Santini & Ruffo (2003): ATL, Atlantic Sea (Iberian, French and British coasts) and NATL, North Atlantic Sea (Norwegian and Arctic regions), so that our final distribution comprised nine different categories.

Network analysis

To provide insights into the biogeographic distribution of amphipod assemblages, a thresholding approach was used in order to identify groups of localities having stronger similarity in

terms of community composition (Kivelä, Arnaud-Haond & Saramäki, 2015). Data were ordered as a species/incidence matrix, whose entries represent the presence of species (rows) at each locality (columns). A weighted network, where localities represent nodes and links the strength of connections, was built using the Jaccard index on the species/incidence matrix, to derive a similarity distance matrix of species co-occurrence. Values ranged from 0, when two localities were identical in amphipod composition, to 1, when they shared no taxa, so that links with higher weights indicated low similarity between localities, and vice versa. The thresholding approach was then applied to identify closely related localities by finding the critical value describing the threshold similarity among pairs (i.e. percolation network).

Percolation networks are becoming increasingly used in ecological studies since they allow identifying relationships among nodes (i.e., populations, species, critical scales in landscape ecology) with the advantage of not requiring any *a priori* knowledge of a threshold value (Rozenfeld et al., 2008; Fletcher et al., 2013; Bellisario, 2018). This value was measured by removing distances following a decreasing order (i.e., most dissimilar localities), until the network reaches the threshold value beyond which it becomes fragmented into disconnected clusters. The identification of this value is obtained by calculating the average cluster size $\langle L \rangle$, that is, the average number of localities belonging to an l -size cluster, as a function of the last threshold distance value beyond which links were removed (Stauffer & Aharony, 1992):

$$\langle L \rangle = \frac{1}{N} \sum_{l < l_{\max}} l^2 n_l \quad (1)$$

where N is the total number of localities not included in the largest cluster (l_{\max}) and n_l is the number of clusters containing l localities. Basically, each time a distance value is removed from the network, localities are redistributed in clusters of different sizes, from largest to smallest. This procedure is therefore iterated until the critical threshold is identified in the transitional region characterized by a strong decrease in $\langle L \rangle$, where the network becomes disconnected (for more information about percolation theory, refer to Stauffer & Aharony, 1992). Here, we used the methodology described in Rozenfeld et al. (2008) and implemented in the package ‘sidier’ (Muñoz-Pajares, 2013) of R (R Development Core Team, 2013).

Modularity

After identifying the minimum set of pairwise similarities between localities, we tested for the presence of a significant pattern of aggregation between localities, and if this pattern reflected a geographic component. To this end, we measured the modularity (Q), which is defined as the degree to which a network can be subdivided in aggregated sets of nodes (i.e., modules), where the within-module links are significantly higher than between-module ones (Newman & Girvan, 2004; Fortunato, 2010). Modularity provides a formal description of the pattern of aggregation between species, populations or communities, being able to identify critical scales in specific ecological and evolutionary processes (Fletcher et al., 2013). Modularity was measured by using the equation originally described by Newman and Girvan (2004):

$$Q = \frac{1}{2m} \sum_{i,j} [A_{ij} - P_{ij}] \delta(C_i, C_j) \quad (2)$$

where m is the total number of links in the percolation network (see above), A_{ij} is the matrix expressing the degree of similarity between localities i and j , $\delta(C_i, C_j)$ is a matrix indicating whether i and j are members of the same module and P_{ij} is the probability in the null model that a link exists between i and j . The extent to which links are distributed within and among modules was tested against an appropriate null model, to correct the observed value of Q by null model expectation. Here we used a simulated annealing algorithm (SA) to test for the significance of a modular partitioning by generating 1,000 null matrices having the same degree distribution as the original network. Under the SA algorithm, affiliation of nodes to modules has an accuracy of 90%, and a significant modular structure was found if the empirical Q value lies above the 95% confidence interval for Q in the randomized networks (Guimerà & Amaral, 2005). Starting from the modular partition, we further assigned the role of each locality in the network by using two topological measures related to the number of species of the local fauna (l , local topological richness) and the distribution of its associated species to other modules (r , regional topological linkage) (Carstensen et al., 2012). The two-dimensional space given by l - r allows the subdivision of localities in: peripherals, few local and regional species; non-hub connectors, few local and many regional species; provincial hubs, many local and few regional species; connector hubs, many local and regional species (revised after Carstensen et al., 2012). Following Carstensen et al. (2012), non-hub localities (i.e., peripherals and connectors) can be interpreted as sink, able to receive species from source localities both within their

own module and of other modules. Conversely, hub-localities (i.e., provincial and connector hubs) can be interpreted as source for both their modules (module hubs) and the entire network (network hubs). As links in our network relates with pattern of similarity between assemblages, the role of localities allowed for a straightforward description of how amphipod diversity could have spread between different areas of the Mediterranean basin.

Multivariate analysis

To explore to what extent the measured network characteristics (i.e., modularity and nodes topology) were related to the biogeographic distribution of amphipods, we ran a between-group correspondence analysis (BGCA) on the ‘sites x species-biogeographic classes’ matrix, where groups were given by the identified modules. BGCA performs a classic Correspondence Analysis (CA) of the per-group centres of gravity, providing an ordination of the groups by maximizing the between-group variance (Baty et al., 2006). From the nine species-biogeographic classes derived from the literature (see above), data were aggregated by summing the number of species belonging to each class in a given site.

3.4 RESULTS

The final dataset obtained from literature showed that to date 152 amphipod species belonging to 77 genera have been identified in *P. oceanica* meadows from 28 Mediterranean localities (Supplemental Materials & Methods S1).

Modules identification

The percolation network showed a co-occurrence similarity threshold of 0.74, which means that sampling sites are expected to share no more than 74% of amphipod species. This led to a network structure of 28 localities joined by 104 links, showing a significant modular structure when

compared with randomized models ($Q = 0.466 \pm 0.005$, $P < 0.001$). Four distinct modules were identified, characterized by a clear geographic distribution: Central-Western Mediterranean (CWM), Tunisian (TUN), Ionian (ION) and Adriatic (ADR) (Fig. 2a).

Eleven localities constituted the Central-Western Mediterranean module (CWM), which spanned from the Spanish coasts till the southern Tyrrhenian ones, including Lampedusa (LAM) and Marettimo (MART) Islands. This module contained the highest number of species (133), of which a high percentage were module exclusives (i.e., present in a single module). Indeed, 85 species were exclusively linked (i.e., observed) to the CWM module, which shared 20, 11 and 2 species with the TUN, ION and ADR modules, respectively, while 14 are in common among all the four modules (Fig. 2b). The CWM module also showed the highest number of Mediterranean endemics (*sensu* Bellan-Santini & Ruffo, 2003) as, for example, species belonging to the genus *Peltocoxa* (*P. gibbosa*, *P. mediterranea*, *P. marioni*) (Fig. 2b, Table 1 and Table S2 in Supplemental Materials & Methods S1).

The Tunisian module (TUN) comprised all 9 localities belonging to the Tunisian coasts and showed both a high number of species and a high percentage of module exclusives, as for example the species belonging to the genus *Elasmopus* (*E. brasiliensis*, *E. pecteniscrus*, *E. pocillimanus*). This module was also characterized by the lowest number of Mediterranean endemics (Fig. 2b, Table 1 and Table S2 in Supplemental Materials & Methods S1).

Both the Adriatic (ADR, 3 localities) and Ionian (ION, 3 localities) modules were characterized by having a few species and a low percentage of module exclusives (Fig. 2b and Table 1). The ION module showed some Mediterranean endemics like *Iphimedia minuta* or *Maera pachytelson*, whilst the ADR module showed a high percentage of cosmopolitan species as for example *Leucothoe spinicarpa*.

The topological role of localities has been assessed by modularity analysis (Fig. 2c), so that each locality has been assigned to a category according to the topological linkage, i.e., local or regional. Localities with a few, local (i.e., module exclusive) species are considered as peripheral nodes (R1 in Fig. 2c), while nodes with a high number of species characterized by regional distribution (i.e., shared among many modules) are considered as connector hubs (R4 in Fig. 2c). Localities in the ADR and ION modules were all classified as peripherals or non-hub connectors (R1 and R2 in Fig. 2c), meaning that amphipod assemblages in these localities are composed by few local species and by a higher (although not very consistent) number of species having a regional distribution (see Materials &

Methods). Localities in the CWM and TUN modules were classified mainly as hubs, subdivided between provincial and connector hubs (R3 and R4 in Fig. 2c). More than half (54%) of localities in the CWM module can be considered connector hubs, characterized by many local and regional species, while most of localities in the TUN module were classified as provincial hubs, so having a larger number of local than regional species (Fig. 2c). The highest values of species diversity are found in hub localities (R3 and R4), while the lowest values of diversity were found in peripheral localities (R1 and R2) together with some hubs, as the 9 TUN localities (Fig. 2c).

Multivariate pattern

The first two principal dimensions of the BGCA accounted for almost 80% of the total inertia (Fig. 3), showing that some biogeographic groups of species concur in explaining the observed pattern of between-modules variance (Fig. 3). Mediterranean endemic species (ME) accounted for almost 50% of the total variance explained by the first dimension of the BGCA, which was associated with the pattern of distribution of assemblages in the CWM module. Indo-Pacific (INDP) and African (AFR) species accounted for more than 30% of variance along the first dimension (Fig. 3), providing a clear differentiation of the TUN module from all others. Cosmopolitan species (COSMP) accounted for 20% of the between-module variance explained by the second dimension of the BGCA, characterizing the pattern of ordination of the ADR module (Fig. 3). With respect to the biogeographic role of localities, provincial and connector hubs seemed to be characterized by both Mediterranean endemics (ME) and species of Indo-Pacific distribution (INDP), while peripheral localities were characterized mainly by cosmopolitan species (COSMP), although the overall pattern was not sharply defined (Fig. 3).

3.4 DISCUSSION

Modules are biogeographically based

Our findings showed that amphipod assemblages are heterogeneous throughout the Mediterranean area, with a maximum of 74% of species shared among localities, and that these differences lay on a geographical base. Despite the network approach does not relay on spatial information, the identified modules correspond to four geographic regions of the Mediterranean Sea: Central-Western Mediterranean (CWM), Tunisian (TUN), Ionian (ION) and Adriatic (ADR). Each region is delimited by well-known barriers, such as the Almeria-Oran Front, the Sicily Channel and the Strait of Otranto (highlighted as n. 2, 5 and 6 in Fig. 1). All these barriers have been pointed out as the most relevant in accounting for by ecological and biogeographic heterogeneity across the Mediterranean Sea, and all of them set a quite abrupt change in salinity and temperature regimes of adjacent basins.

The Almeria-Oran Front (AOF) is the western boundary of the Central-Western module (CWM), corresponding to the range boundary of the Mediterranean endemic *P. oceanica*, unable to tolerate the low temperature and salinity of the Alboran Sea (Boudouresque, 2004). Circulation patterns and changes in temperature and salinity across the Sicily Channel, both concur in partially preventing the dispersal of a number of species across the threshold of the Siculo-Tunisian Straits (Robinson et al., 1992; Coll et al., 2010). In our study, this is the divide between the CWM and TUN modules, with this latter grouping the localities along the Tunisian coasts characterized by the presence of the jet-like Algerian Current and Atlantic Ionian Stream (Pinardi & Masetti, 2000). The Strait of Otranto delimits the Adriatic Sea, a semi-enclosed basin where several factors, as winds, tides and freshwater runoff from rivers, all determine peculiar low salinity and low winter temperatures (Falco et al., 2000; Lejeusne et al., 2010). This is the boundary between the ADR and ION modules, which moderately exchange water mass through the Albanian side of the Strait of Otranto (Orlic, Gacic & Laviolette, 1992).

The boundaries between modules are represented by the most effective Mediterranean barriers, in agreement with the geographic patterns highlighted in other organisms studied at the whole basin scale. The areas corresponding to the Western and Eastern Mediterranean and to the Adriatic Sea have been historically considered as different biogeographic provinces, hosting differentiated species

assemblages of macrophytes, diatoms and many animal groups (Ignatiades et al., 2009; Gambi, Lampadariou & Danovaro, 2010). Among invertebrates, an analysis of the Mediterranean sponge regional diversity showed distinct assemblages from CW Mediterranean, Tunisia, Adriatic and Ionian Sea (Gerovasileiou & Voultsiadou, 2012). Similar results were reported for benthic polychaetes, showing different assemblages in the Western and Central Mediterranean basins and in the Adriatic Sea (Arvanitidis et al., 2002), while deep-sea megafauna showed dissimilar community compositions in Western Mediterranean and Ionian basins (Tecchio et al., 2010).

Modules diversity and species distribution

The differentiation among the four modules depends on different aspects of assemblage diversity and composition. The assemblages characterized by higher diversity were all from the CWM module, and in particular were located in the Central Tyrrhenian area (e.g., CHIA, MOR, GIAN, TAV; Fig. 2b). The localities from ADR and ION modules showed the lowest diversity values, together with a few localities from CWM (MART, MED) and TUN (ZAP, RAF). A decreasing gradient in species richness from north-west to south-east is a generalized pattern in the Mediterranean Sea, with an overall animal species diversity 100% greater in the western than in the eastern basin in both vertebrates and invertebrates (Boudouresque, 2004; Coll et al., 2010). As an example, the diversity of deep-sea nematode assemblages decreases with depth but, when similar depths are compared, a longitude effect appears, with diversity decreasing eastward (Danovaro et al., 2008). A similar pattern was detected in deep-sea foraminifers, whose species richness decreases from western to eastern Mediterranean, likely mimicking the longitudinal cline of organic matter availability on the deep seafloor (Danovaro et al., 2010).

The biogeographic role of localities showed how assemblages differ among and within modules, by identifying hubs *vs.* peripheral localities. Localities with assemblages having a high number of species characterized by regional distribution (i.e., shared among many modules) are considered as connector hubs and are supposed to behave as source in a biogeographic context. On the opposite, localities where assemblages contain few, local species are labelled as peripheral nodes, and considered as sinks. Interestingly, nearly all the localities of CWM and TUN modules are classified

as hubs, meaning that they share a large proportion of species with many other localities belonging to other modules, besides a relevant number of species among each other. On the contrary, all the localities from ADR and ION modules are considered as peripherals or ultra-peripherals, so having assemblages very similar at intra-module level and highly differentiated with respect to localities belonging to other modules. Both modules are characterized by a small number of module exclusive species, together with a sub-set of species found either in the whole basin or in the hub localities of the CWM module.

The identified modules differ not only in species richness and biogeographic roles of their associated localities, but also in the biogeographic distribution of the species found in various assemblages. The pattern recovered links species' richness and distribution, with low-richness modules characterized by species having wider distributions, and may be explained in the light of biogeographic considerations. For instance, Indo-Pacific and African species associated with warm waters typify the rich Tunisian assemblages, as for example those belonging to the genus *Elasmopus* (Fig. 3). Mediterranean endemics and, to a lesser extent, North-Atlantic species characterize the high diversity of CWM. *Apherusa chiereghinii*, *Cressa cristata*, *Gammaropsis crenulata*, and the species belonging to the genus *Peltocoxa* are examples of Mediterranean endemics exclusive of the CWM module, while some species belonging to the genus *Ampelisca* (*A. sarsi*, *A. serraticaudata* and *A. tenuicornis*) represent an example of temperate species of Atlantic origin inhabiting also the western part of the Mediterranean Sea. This pattern is due to the well-known paleogeographic history of the basin, with particular regard to the most recent cycles of Plio-Pleistocene glaciations (Coll et al., 2010). These climatic oscillations induced temperate Atlantic species to periodically enter the Mediterranean Sea where they originated vicariant endemic species as a result of geographic isolation and local adaptation. Examples are species belonging to the genera *Apherusa*, *Tritaeta* and *Tmetonyx*, which represent the cold component of the Mediterranean amphipod fauna (Bellan-Santini & Ruffo, 2003). For this reason the Mediterranean Sea is considered as a 'diversity pump' from the Atlantic and the identification of its biogeographic provinces largely rely on the distribution of Mediterranean endemics (Bianchi & Morri, 2000; Bianchi et al., 2012). Similarly, it has been shown that species originated from warm faunas prevail in the south-eastern part of the Mediterranean basin (Lejeusne et al., 2010). Accordingly, the presence of warm species as a representative of the Tunisian coasts has been signalled for other invertebrates, e.g., sponges (Gerovasileiou & Voultsiadou, 2012).

Assemblages in the geographically confined ADR module are characterized mainly by widely distributed species with cosmopolitan range. This observation, and the fact that only few species are found exclusively in this module, both suggest that this area may be particularly difficult to be colonized and behave as a sink, as shown by modularity analysis (Fig. 3c). This difficulty can be related to both extreme environmental conditions and geographic isolation, so that only vagile and tolerant species may enter and establish in this basin. Indeed, the Adriatic Sea is characterized by low salinity and winter temperatures, together with a moderate water mass exchange with the neighbouring Ionian Sea through the Strait of Otranto (Orlic, Gacic & Laviolette, 1992; Falco et al., 2000). Moreover, the Adriatic basin was largely dried during the Last Glacial Maximum (LGM, 23K-19K years ago; Rohling et al., 2010), so that a significant part of the Adriatic fauna has only recently re-colonized this basin. Within the poorly vagile group of amphipods, which are brooding species lacking a pelagic larval stage, cosmopolitan species are generally eurhyaline, eurytherm and more prone to passive dispersal (Bellan-Santini & Ruffo, 2003), thus having the highest probability to reach and settle in the Adriatic habitat. Accordingly, a recent checklist of opisthobranch Adriatic fauna signalled that the great majority of species had an Atlantic-Mediterranean range, while only few were Mediterranean endemics (Zenetos et al., 2016).

Amphipod diversity and paleogeographic history of *Posidonia oceanica*

Populations of *P. oceanica* inhabiting the western and eastern parts of the Mediterranean Sea are genetically differentiated, with those from the central Mediterranean around the Siculo-Tunisian Strait characterized by a higher genetic diversity (Arnaud-Haond et al., 2007; Serra et al., 2010). This pattern was interpreted as the result of a secondary contact zone between the western and eastern forms, each one originated by vicariance in glacial refugia during the Last Glacial Maximum (LGM) (Serra et al., 2010). A more recent study has superimposed Ecological Niche Modelling to phylogeographic data, highlighting the southern Mediterranean as the most climatically suitable area during LGM, with particular regard to the central zone. This area was then proposed as the main glacial refugium of the seagrass, thus explaining its higher genetic diversity as due to the long-term persistence in this region (Chefaoui, Duarte & Serrão, 2017).

Glacial refugia have been repeatedly pointed out as hot-spots and melting pots of diversity, not only in terrestrial environments but also in marine habitats (Hewitt, 1999; 2004; Maggs et al.,

2008). This diversity may concern both genetic lineages and community richness, in agreement with the postulated relationship between habitat stability and community diversity (Hewitt, 2000; Ives & Carpenter, 2007). Within this frame, the high diversity and the hub role of assemblages in TUN module can be explained by their localization in a glacial refuge area, as proposed by Chefaoui, Duarte & Serrão (2017). However, the same pattern characterized the CWM assemblages, which are richer in species and share an even greater percentage of their species with other modules. This finding however may be only apparently in contrast with the lower presence probability of *P. oceanica* in the northern Mediterranean during LGM, if we consider that amphipods living on *P. oceanica* are not exclusively found in this habitat.

Indeed, Amphipods can actively choose their substratum, and this habitat preference produces differences in their abundance on various seagrasses and other substrates, rather than a presence/absence pattern (Sanchez-Jerez et al., 1999; Vázquez-Luis, Sanchez-Jerez & Bayle-Sempere, 2009). A possible scenario is therefore that the northward seagrass range expansion triggered by climate warming after LGM prompted the migration of part of the associated fauna. At the same time, the north-western Mediterranean was likely already inhabited by a local pool of amphipod species derived from the Atlantic (according to the ‘biodiversity pump’ mechanism), therefore adapted to temperate climatic conditions and able to survive during LGM. Under this hypothesis, it is expected that the CWM assemblages would include many module endemics represented by species with Mediterranean and/or Atlantic distribution (i.e., ME and ATL according to biogeographic classes). Also, CWM and TUN modules should share a relevant number of species (i.e., those originated in the southern refuge and migrated northward with the seagrass). Our analyses showed that all these expectations were verified, supporting the blending between resident, temperate species with the warm-adapted ones spreading from the south with *P. oceanica* during LGM in explaining the current richness of amphipod fauna in CWM.

3.5 CONCLUSIONS

To our knowledge, this study is the first trying to assess the pattern of co-occurrence of Mediterranean benthic amphipod assemblages associated with a peculiar habitat, such as *P. oceanica* meadows. A network approach based on modularity has proven useful in detecting the biogeographic subdivisions of assemblages and in assessing biogeographic roles of associated localities. Our results provide a new perspective on the less studied southern Mediterranean, which may gain a relevant place in the origin of the basin biodiversity as a LGM refuge, besides confirming the known role of the Central Western Mediterranean area as a ‘biodiversity pump’ from the Atlantic. Our findings also suggest how the distribution of amphipod diversity in *P. oceanica* meadows stems from complex interactions between present and past geographic barriers, local species adaptation, and the biogeography of the host plant.

To this end, comparative phylogeographical studies can be used to verify the assumptions made in this study, in order to clarify the history of the biogeographical modules identified. This can open the road to a series of studies aimed at deepening the knowledge of the most common and abundant species shared by modules, to identify the centre of evolution and/or dispersion of amphipods within the Mediterranean. For instance, a comparative phylogeography between congeneric species shared by the TUN and CWM module but showing different distributions (e.g., *Apolochus*, *Ampithoe*, *Dexamine*) can help both disentangling the role of the southern basin in the diversity of amphipods, and testing for the hypothesis of a convergence between the ecological and evolutionary outcomes of amphipods and seagrass in the Mediterranean basin.

3.6 TABLES AND FIGURES

Table 1 - Modular subdivision of sampling localities. l is the number of localities in each module; s the total number of species. Module endemics is the percentage of species exclusively present in a single module; ME and COSMP are the percentage of species in each module belonging to the Mediterranean Endemics (ME) and Cosmopolite (COSMP) biogeographic classes (see Materials and Methods).

Module	l	s	Module endemics (%)	Mediterranean endemics (ME%)	Mediterranean cosmopolite (COSMP%)
CWM	11	133	64.7	16.7	4.8
TUN	9	47	27.6	7.3	7.3
ION	3	26	15.4	13	4.3
ADR	3	11	18.4	9	27.3

Figure 1 - Overview of the Mediterranean Sea with reference localities (for acronyms, see Table A1 in Supplementary Materials). Arrows indicate the main circulation patterns and red lines the main barriers. 1, Gibraltar Strait; 2, Almeria-Oran Front; 3, Ibiza Channel; 4, Balearic Front; 5, Sicily Channel; 6, Otranto Strait.

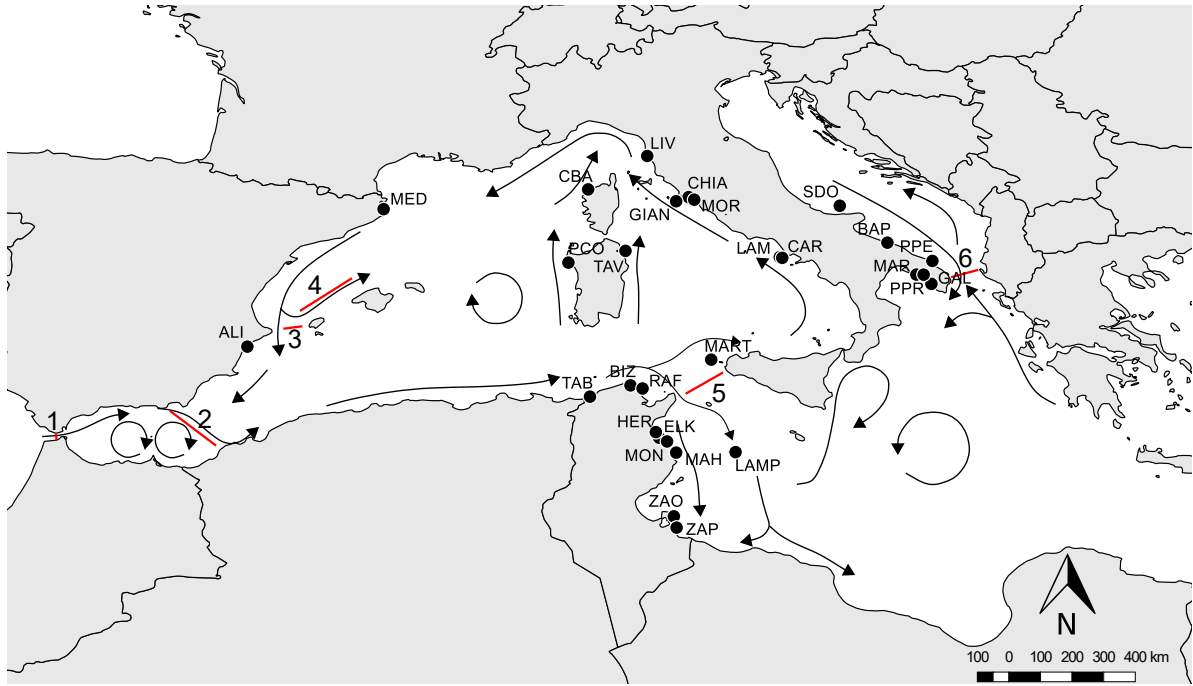


Figure 2 - a: spatial distribution of the modular subdivision of localities, with colours indicating different modules. b: visualization of biogeographic modules and associated amphipod species (black dots). c: plot showing the biogeographic role of localities in the topological space given by local topological linkage (l) and regional topological linkage (r) (revised after Carstensen et al. 2012). Colors of localities correspond to the identified modules, with size proportional to species diversity. R1, Peripheral nodes: few local and regional species; R2, Non-hub connector nodes: few local and many regional species; R3, Provincial hubs: many local and few regional species; R4, Connector hubs: many local and regional species.

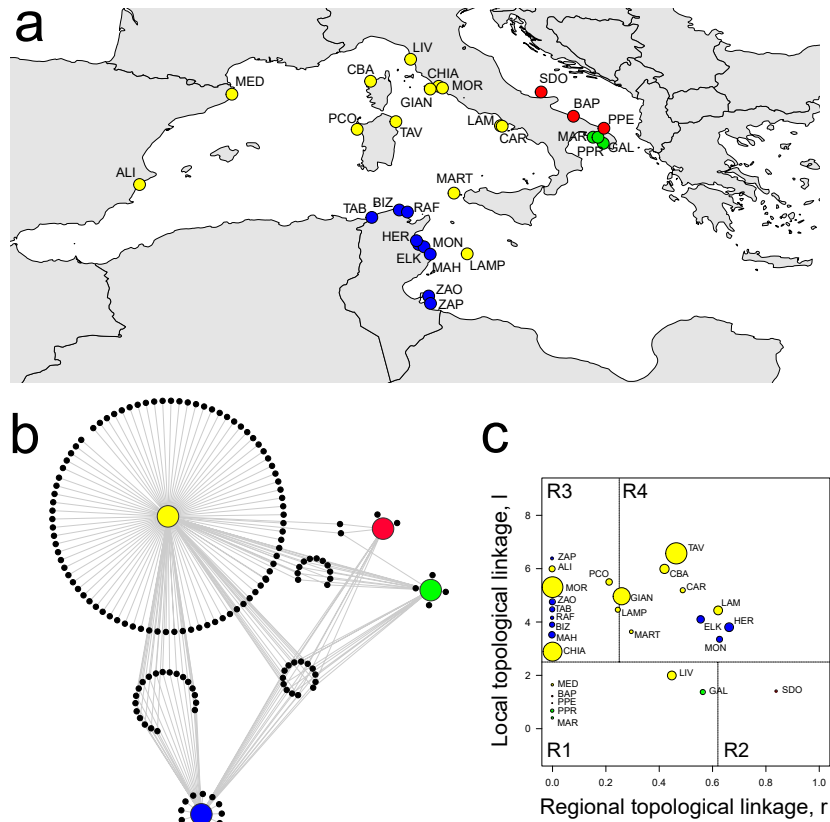
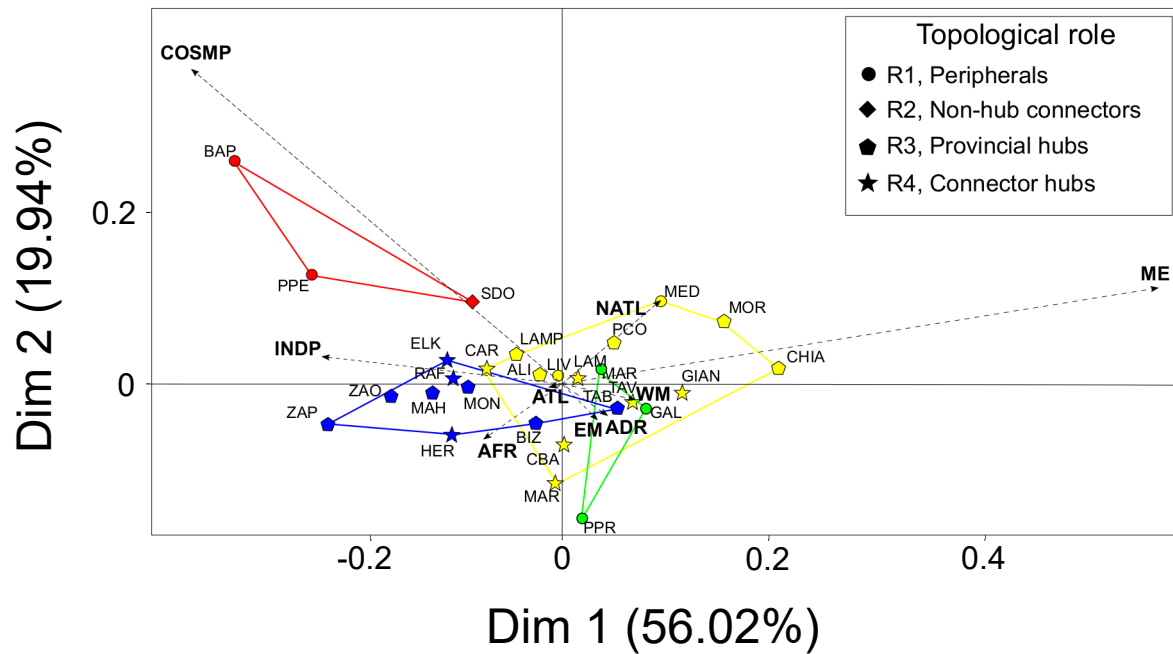


Figure 3 - Between-Group Correspondence Analysis (BGCA) of localities based on the nine species-biogeographic classes according to literature. Colors indicate the identified modules and different shapes correspond to the biogeographic role of localities (see top-right box). Dashed arrows indicate the nine species-biogeographic classes: COSMP, Cosmopolite; INDP, Indo-Pacific; AFR, African coasts from Ceuta to Cap Vert; ATL, Atlantic coasts from Spain to Britain; NATL, North Atlantic from Norway to Arctic Sea; WM, West Mediterranean; EM, East Mediterranean; ADR, Adriatic Sea; ME, Mediterranean endemics.



Supplemental Materials & Methods S1

An extensive (meta) analysis of the current and past literature was conducted in order to extract all available information about the distribution of amphipods on *Posidonia oceanica* at Mediterranean basin level. We identified Summer (June to September) as the most frequent sampling period and a depth of 0.5-30 m as the most frequent range.

Unfortunately, no common sampling procedures were found in different studies, so that we focused on those able to sample (at least) motile organisms living in the leaf stratum. The final dataset was thus built by standardizing the information deriving from three main sampling procedures, briefly described below:

- i) Air-lift sampling: is a technique originally described in Bussers et al. (1983) where an air-lift sampler with a 500- μ m mesh size is used to suck up at constant airflow (for at least 2 minutes) samples from the meadows. The sampling surface, with three replicates, is delimited by a 40×40 cm quadrant (Michel et al., 2010).
- ii) Hand-towel net: originally described in Russo et al. (1985), consists in a series of strokes to shake the leaves of *P. oceanica* from the basis, with the net (400- μ m mesh size) held to form an angle of ca. 45° with the bottom (Bedini et al., 2011; Scipione et al., 1996).
- iii) Clumps collection: a technique where a quadrant of 30 cm side and 25 cm height was placed in each *Posidonia* meadow. Shoots are pulled up and collected in a bag with a net size of 0.3 mm, and species are subsequently sorted and identified in the lab.

In order to further homogenize the different datasets, we decided to retain only information about the presence/absence of amphipods on *Posidonia oceanica* meadows. Abundance data were only used to select the presence of a given species, removing singletons from the original dataset.

Overall, the final dataset contains 28 localities (Table S1), of which 9 located in Tunisia, 18 in Italy (Tyrrhenian, Adriatic and Ionian Sea), 2 in Spain and 1 in Corsica (France) (see Fig.1 in the main text), for a total of 152 species (Table S2). Since data come from different Authors, a possible problem

concerning species nomenclature may raise. However, all the Authors used the reference taxonomic keys by Ruffo (1982-98) and, for more recent works, updated their nomenclature according to the World Register of Marine Species (WoRMS). This warrants for consistency in species identification and allowed us to uniformly update nomenclature according to WoRMS (last access 17/01/2019).

Table S1 - Geographic location of sampling localities, associated acronyms and reference literature.

Country	Locality	Acronym	Reference literature
Italy	Isola di Marettimo	MART	Scipione, 1999
	Isola di Lampedusa	LAMP	Scipione, 1999
	Lacco Ameno	LAM	Scipione, 1999
	Castello Aragonese	CAR	Scipione, 1999
	Punta Morelle	MOR	Bellisario et al., 2015
	Chiarone	CHIA	Bellisario et al., 2015
	Isola di Giannutri	GIAN	Bellisario et al., 2015
	Livorno	LIV	Bedini et al., 2011
	Calvi Bay	CBA	Sturaro et al., 2015
	Porto Conte	PCO	Scipione, 1999
	Isola di Tavolara	TAV	Sturaro et al., 2014
	San Domino	SDO	Scipione, 1999
	Bari Palese	BAP	Diviacco, 1998
	Punta Penne	PPE	Diviacco, 1998
	Gallipoli	GAL	Diviacco, 1998; Scipione, 1999
	Punta Prosciutto	PPR	Diviacco, 1998
	Maruggio	MAR	Diviacco, 1998
Spain	Medes Island	MED	Scipione, 1999
	Alicante	ALI	Sánchez-Jerez et al., 2000
Tunisia	Tabarka	TAB	Zakhama-Sraieb et al., 2006; 2010
	Bizerte	BIZ	Zakhama-Sraieb et al., 2006; 2010
	Rafraf	RAF	Zakhama-Sraieb et al., 2006; 2010
	Hergla	HER	Zakhama-Sraieb et al., 2006; 2010
	El Kantoui	ELK	Zakhama-Sraieb et al., 2006; 2010
	Monastir	MON	Zakhama-Sraieb et al., 2006; 2010
	Mahdia	MAH	Zakhama-Sraieb et al., 2006; 2010
	Zarzis Ogla	ZAO	Zakhama-Sraieb et al., 2006; 2010
	Zarzis Plage	ZAP	Zakhama-Sraieb et al., 2006; 2010

Table S2 - List of amphipod species recovered from the literature, with their presence/absence in different biogeographic modules. TUN, Tunisia; ION, Ionian Sea; ADR, Adriatic Sea; CWM, Central Western Mediterranean Sea.

	TUN	ION	ADR	CWM
<i>Abludomelita aculeata</i> (Chevreux, 1911)	0	0	0	1
<i>Abludomelita gladiosa</i> (Spence Bate, 1862)	0	0	0	1
<i>Acidostoma obesum</i> (Spence Bate, 1862)	0	1	0	0
<i>Ampelisca antennata</i> Bellan-Santini & Kaim-Malka, 1977	0	0	0	1
<i>Ampelisca diadema</i> (Costa, 1853)	0	1	1	1
<i>Ampelisca pseudospinimana</i> Bellan-Santini & Kaim-Malka, 1977	0	0	0	1
<i>Ampelisca rubella</i> A. Costa, 1864	1	0	0	1
<i>Ampelisca sarsi</i> Chevreux, 1888	0	0	0	1
<i>Ampelisca serraticaudata</i> Chevreux, 1888	0	0	0	1
<i>Ampelisca spinipes</i> Boeck, 1861	1	0	0	1
<i>Ampelisca tenuicornis</i> Liljeborg, 1855	0	0	0	1
<i>Ampelisca unidentata</i> Schellenberg, 1936	1	0	0	1
<i>Amphilochus brunneus</i> Della Valle, 1893	0	0	0	1
<i>Ampithoe ferox</i> (Chevreux, 1901)	0	0	0	1
<i>Ampithoe ramondi</i> Audouin, 1826	1	1	0	1
<i>Apolochus neapolitanus</i> (Della Valle, 1893)	1	1	0	1
<i>Apolochus picadurus</i> (J. L. Barnard, 1962)	0	1	0	1
<i>Animoceradocus semiserratus</i> (Spence Bate, 1862)	0	0	0	1
<i>Aora gracilis</i> (Spence Bate, 1857)	1	0	0	1
<i>Aora spinicornis</i> Afonso, 1976	1	1	1	1
<i>Apherusa alacris</i> Krapp-Schickel, 1969	0	0	0	1
<i>Apherusa bispinosa</i> (Spence Bate, 1857)	0	0	0	1
<i>Apherusa chiereghinii</i> Giordani-Soika, 1949	0	0	0	1
<i>Apherusa vexatrix</i> Krapp-Schickel, 1979	0	0	0	1
<i>Apocorophium acutum</i> (Chevreux, 1908)	1	0	0	1
<i>Autonoe spiniventris</i> Della Valle, 1893	0	1	0	1
<i>Caprella acanthifera</i> Leach, 1814	1	0	0	1
<i>Caprella equilibra</i> Say, 1818	0	0	0	1
<i>Caprella grandimana</i> (Mayer, 1882)	1	0	0	1
<i>Caprella liparotensis</i> Haller, 1879	1	0	0	1
<i>Caprella rapax</i> Mayer, 1890	0	0	0	1
<i>Caprella tavolarenensis</i> Sturaro & Guerra-García 2012	0	0	0	1
<i>Centraloecetes dellavallei</i> (Stebbing, 1899)	0	0	0	1
<i>Centraloecetes neapolitanus</i> (Schiecke, 1978)	0	0	0	1

<i>Ceradocus</i> (<i>Ceradocus</i>) <i>orchestiipes</i> Costa, 1853	0	1	0	1
<i>Cheirocratus sundevallii</i> (Rathke, 1843)	0	0	0	1
<i>Coboldus nitior</i> Krapp-Schickel, 1974	0	0	0	1
<i>Cressa cristata</i> Myers, 1969	0	0	0	1
<i>Cressa mediterranea</i> Ruffo, 1979	0	0	0	1
<i>Cymadusa crassicornis</i> (Costa, 1853)	0	0	0	1
<i>Deflexilodes gibbosus</i> (Chevreux, 1888)	0	0	0	1
<i>Deflexilodes griseus</i> (Della Valle, 1893)	0	0	0	1
<i>Deflexilodes subnudus</i> (Norman, 1889)	0	0	0	1
<i>Deutella shieckei</i> Cavedini, 1982	1	0	0	0
<i>Dexamine spiniventris</i> (Costa, 1853)	1	1	0	1
<i>Dexamine spinosa</i> (Montagu, 1813)	1	1	1	1
<i>Elasmopus brasiliensis</i> (Dana, 1855)	1	0	0	0
<i>Elasmopus pecteniscrus</i> (Spence Bate, 1862)	1	0	0	0
<i>Elasmopus pocillimanus</i> (Spence Bate, 1862)	1	0	0	0
<i>Elasmopus rapax</i> Costa, 1853	0	0	0	1
<i>Erichthonius brasiliensis</i> (Dana, 1853)	1	0	1	1
<i>Erichthonius difformis</i> H. Milne Edwards, 1830	1	0	0	0
<i>Erichthonius punctatus</i> (Spence Bate, 1857)	1	1	0	1
<i>Eusiroides dellavallei</i> Chevreux, 1899	1	0	0	1
<i>Gammarella fucicola</i> (Leach, 1814)	1	1	0	1
<i>Gammarellus angulosus</i> (Rathke, 1843)	0	0	0	1
<i>Gammaropsis crenulata</i> Krapp-Schickel & Myers, 1979	0	0	0	1
<i>Gammaropsis dentata</i> Chevreux, 1900	1	0	0	1
<i>Gammaropsis maculata</i> (Johnston, 1828)	0	0	0	1
<i>Gammaropsis ostroumowi</i> Sowinski, 1898	1	0	0	0
<i>Gammaropsis palmata</i> (Stebbing & Robertson, 1891)	0	1	0	1
<i>Gammaropsis ulrici</i> Krapp-Schickel & Myers, 1979	0	0	0	1
<i>Gammarus aequicauda</i> (Martynov, 1931)	0	0	0	1
<i>Gammarus crinicornis</i> Stock, 1966	0	0	0	1
<i>Gitana sarsi</i> Boeck, 1871	0	0	0	1
<i>Guernea</i> (<i>Guernea</i>) <i>coalita</i> (Norman, 1868)	0	0	0	1
<i>Harpinia crenulata</i> (Boeck, 1871)	0	0	0	1
<i>Harpinia zavodniki</i> Karaman, 1987	0	0	0	1
<i>Hyale camptonyx</i> (Heller, 1866)	1	0	0	1
<i>Hyale crassipes</i> (Heller, 1866)	0	0	0	1
<i>Hyale schmidtii</i> (Heller, 1866)	0	1	0	1
<i>Hyale stebbingi</i> Chevreux, 1888	0	0	0	1
<i>Iphimedia minuta</i> G. O. Sars, 1883	0	1	0	1
<i>Iphimedia serratipes</i> Ruffo & Schiecke, 1979	0	0	0	1
<i>Iphimedia vicina</i> Ruffo & Schiecke, 1979	0	0	0	1
<i>Ischyrocerus inexpectatus</i> Ruffo, 1959	1	0	0	1
<i>Jassa oia</i> (Spence Bate, 1862)	0	0	0	1
<i>Kroyera carinata</i> Spence Bate, 1857	0	0	0	1

<i>Lembos websteri</i> Spence Bate, 1857	1	0	0	1
<i>Lepidepcreum longicornis</i> (Spence Bate 1862)	1	0	0	1
<i>Leptocheirus bispinosus</i> Norman, 1908	0	0	0	1
<i>Leptocheirus guttatus</i> (Grube, 1864)	1	0	0	1
<i>Leptocheirus longimanus</i> Ledoyer, 1973	0	0	0	1
<i>Leptocheirus pectinatus</i> (Norman, 1869)	0	1	0	1
<i>Leptocheirus pilosus</i> Zaddach, 1844	0	0	0	1
<i>Leucothoe euryonyx</i> (Walker, 1901)	0	0	0	1
<i>Leucothoe procera</i> Spence Bate, 1857	0	0	0	1
<i>Leucothoe richiardii</i> Lesson, 1865	0	0	1	0
<i>Leucothoe spinicarpa</i> (Abildgaard, 1789)	1	0	1	1
<i>Leucothoe venetiarum</i> Giordani-Soika, 1950	1	0	0	0
<i>Liljeborgia dellavallei</i> Stebbing, 1906	0	1	0	1
<i>Lysianassa costae</i> (H. Milne Edwards, 1830)	1	1	0	1
<i>Lysianassina longicornis</i> (Lucas, 1846)	0	0	0	1
<i>Lysianassa pilicornis</i> (Heller, 1866)	0	1	0	1
<i>Lysianassa plumosa</i> Boeck, 1871	0	1	0	0
<i>Lysianella dellavallei</i> Stebbing, 1906	0	0	0	1
<i>Maera grossimana</i> (Montagu, 1808)	0	1	0	1
<i>Maera hironellei</i> Chevreux, 1900	1	0	0	0
<i>Maera pachytelson</i> Karaman & Ruffo, 1971	0	1	0	0
<i>Maera sodalis</i> Karaman & Ruffo, 1971	0	0	0	1
<i>Maerella tenuimana</i> (Spence Bate, 1862)	0	0	0	1
<i>Marinohyalella richardi</i> (Chevreux, 1902)	0	0	0	1
<i>Medicorophium minimum</i> (Schiecke, 1978)	0	0	0	1
<i>Metaphoxus fultoni</i> (Scott, 1890)	0	0	0	1
<i>Metaphoxus simplex</i> (Spence Bate, 1857)	1	0	0	0
<i>Microdeutopus algicola</i> Della Valle, 1893	0	0	0	1
<i>Microdeutopus anomalus</i> (Rathke, 1843)	0	0	0	1
<i>Microdeutopus chelifera</i> (Spence Bate, 1862)	1	0	0	1
<i>Microdeutopus obtusatus</i> Myers, 1973	0	0	0	1
<i>Microdeutopus similis</i> Myers, 1977	0	0	0	1
<i>Microdeutopus stationis</i> Della Valle, 1893	0	0	1	0
<i>Microdeutopus versiculatus</i> (Spence Bate 1857)	0	0	0	1
<i>Microjassa cumbrensis</i> (Stebbing & Robertson, 1891)	0	0	0	1
<i>Micropythia carinata</i> (Spence Bate, 1862)	0	1	0	0
<i>Nannonyx propinquus</i> Chevreux, 1911	0	0	0	1
<i>Nototropis guttatus</i> Costa, 1853	0	0	0	1
<i>Nototropis massiliensis</i> (Bellan-Santini, 1975)	0	0	0	1
<i>Nototropis vedlomensis</i> (Spence Bate & Westwood, 1862)	0	1	0	1
<i>Orchestia montagui</i> Audouin, 1826	0	0	0	1
<i>Orchomene humilis</i> (Costa, 1853)	1	1	0	1
<i>Orchomene similis</i> Chevreux, 1912	0	0	0	1
<i>Othomaera knudseni</i> (Reid, 1951)	0	0	0	1

<i>Parhyale aquilina</i> (Costa, 1857)	1	0	0	0
<i>Pariambus typicus</i> (Krøyer, 1884)	0	0	0	1
<i>Peltocoxa gibbosa</i> (Schiecke, 1977)	0	0	0	1
<i>Peltocoxa marioni</i> Catta, 1875	1	1	1	1
<i>Peltocoxa mediterranea</i> Schiecke, 1977	0	0	0	1
<i>Pereionotus testudo</i> (Montagu, 1808)	1	0	0	1
<i>Periiculodes aequimanus</i> (Korssman, 1880)	0	0	0	1
<i>Periiculodes longimanus</i> (Spence Bate & Westwood, 1868)	0	0	0	1
<i>Photis longicaudata</i> (Spence Bate & Westwood, 1862)	0	0	0	1
<i>Photis longipes</i> (Della Valle, 1893)	0	0	0	1
<i>Phoxocephalus aquosus</i> Karaman, 1985	0	0	0	1
<i>Phthisica marina</i> Slabber, 1769	0	0	1	1
<i>Pleonexes helleri</i> (Karaman, 1975)	1	0	0	1
<i>Podocerus variegatus</i> Leach, 1814	0	0	0	1
<i>Pseudolirius kroyeri</i> (Haller, 1897)	0	0	0	1
<i>Pseudoprotella phasma</i> (Montagu, 1804)	1	0	1	1
<i>Quadrimaera inaequipes</i> (A. Costa in Hope, 1851)	1	0	1	1
<i>Socarnes filicornis</i> (Heller, 1866)	1	0	0	0
<i>Stenothoe dollfusi</i> Chevreux, 1887	0	0	0	1
<i>Stenothoe eduardi</i> Krapp- Schickel, 1975	0	0	0	1
<i>Stenothoe gallensis</i> Walker, 1904	1	0	0	0
<i>Stenothoe monoculoides</i> (Montagu, 1813)	1	0	0	1
<i>Stenothoe tergestina</i> (Nebeski, 1881)	0	0	0	1
<i>Synchelidium longidigitatum</i> Ruffo, 1947	0	0	0	1
<i>Tethylembos viguieri</i> (Chevreux, 1911)	1	0	0	0
<i>Tmetonyx nardonis</i> (Heller, 1867)	0	0	0	1
<i>Tmetonyx similis</i> (G.O. Sars, 1891)	0	0	0	1
<i>Tritaeta gibbosa</i> (Spence Bate, 1862)	1	0	0	1
<i>Unciolella lunata</i> Chevreux, 1911	0	0	0	1
<i>Urothoe poseidonis</i> Reibish, 1905	0	0	0	1

4. CRYPTIC DIVERSITY AND ANCIENT DIVERGENCE IN TWO SPECIES OF AMPHIPODS FROM CENTRAL MEDITERRANEAN SEA

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4.1 ABSTRACT

A phylogeographic analysis was carried out on two species belonging to the genus *Apolochus*, widely distributed in Mediterranean *Posidonia oceanica* meadows. Both mitochondrial (COI) and nuclear (H3) markers were used to verify: 1) if a pattern of high genetic differentiation associated to cryptic diversity is recovered, as recently observed in many marine amphipods; 2) if high haplotype and nucleotide diversity associated to high geographic structuring and to lack of population expansion characterized species structure, so suggesting their survival in localized refugia; 3) if deep or recent splits occurred, with species origin pre- or post-dating the Messinian Salinity Crisis (MSC).

Our results showed: 1) a high cryptic diversity, with six MOTUs (putative species) in the Mediterranean Sea, three in *A. neapolitanus* and three in *A. picadurus* morphospecies. The geographic distribution of these MOTUs showed that at least some of them were able to cross main Mediterranean barriers and to distribute in different seas over the time; 2) a remarkable level of genetic structuring was observed both between and within the different biogeographic areas, suggesting that the levels of ongoing gene flow are low or absent. This geographically structured pattern was coupled with high haplotype and nucleotide diversity and lack of signs of recent expansion at the population level; 3) the diversification within each *Apolochus* morphospecies was ancient, starting during the Terminal Tethyan Event (TTE, 18-12 Mya) and bearing the signature of the main Mediterranean paleogeographic events, such as the MSC (5.9-5.3 Mya) and the onset of ice cycles during Plio-Pleistocene ages (since 3.7 Mya).

These results provided insights into the usefulness of studying poorly vagile marine organisms to assess the biogeographic history of the Mediterranean Sea, since limited dispersal ability allows avoiding the loss of the signature of ancient events because of the superimposition of contemporary high gene flow. Our results suggested that amphipods are among these organisms and that their study may contribute to change the

perception of the Mediterranean biodiversity from having a recent, post-Messinian origin, toward a more ancient derivation thanks to species survival along MSC.

4.2 INTRODUCTION

The Mediterranean Sea is a recognized hot-spot of biodiversity hosting about 17,000 species, which are between 4 to 18% of the global macrofauna, despite it contains only 0.3% of ocean waters (Coll et al., 2010; Bianchi et al., 2012). This high diversity stems from a combination of oceanographic, ecological and biogeographic features allowing the coexistence of species of Atlantic origin with temperate and subtropical organisms (Coll et al., 2010), although it is known that species distribution is also influenced by both past geological events and the dispersal capacity of each species (Provant and Bennett, 2008).

Phylogeographic studies are able to integrate evolution, ecology and historical events to explain species current distribution and their patterns of genetic variation (Avice, 2000). Within the biodiversity hot-spot represented by the Mediterranean Sea, phylogeography has been mainly devoted to study organisms characterized by high vagility, i.e. with high dispersal ability. However, this bias may garble real evolutionary patterns since the contemporary population connectivity could obscure the signature of ancient historical events (Provant and Bennett, 2008; Calvo et al., 2015). As a consequence, this kind of phylogeographic studies highlighted a common and quite recent history made of Plio-Pleistocenic fragmentation into glacial refugia followed by range expansions via postglacial water masses and subsequent secondary contacts among vicariant lineages. This biogeographic scenario however may result unrealistic for poorly vagile species, which are more prone to isolation in fragmented refugia, to local extinction and to high allopatric divergence, which may indirectly result in a high differentiation of populations due to a number of evolutionary process including local selection, inbreeding and drift. While this alternative scenario and its outcomes have been largely studied in terrestrial Mediterranean species (e.g., Cimmaruta et al., 2015), only few studies have been carried out on marine species (Calvo et al., 2015; Liu et al., 2018; Hupalo et al., 2019).

Within this frame, benthic amphipods may be suitable species to test if the mostly supported biogeographic scenarios set up on vagile organisms hold also for these species characterized by low

dispersal ability, being brooding and lacking a pelagic larval stage. We therefore carried out a phylogeographic study on two species belonging to the genus *Apolochus*, which are among the most abundant in *Posidonia oceanica* meadows across the Mediterranean Sea: *A. neapolitanus* and *A. picadurus*. The study area was concentrated in the central Mediterranean Sea, where some of the main biogeographic barriers occur, generating many biogeographic sectors over a relatively small area (Bianchi and Morri, 2000). Stated the features of these species, we have formulated the following hypotheses to be verified in this work: 1) a pattern of high genetic differentiation associated to cryptic diversity is expected, since both morphological stasis and a genetic architecture favoring high speciation rate have been hypothesized in marine amphipods (Richards et al., 2012; Chenouil et al., 2018); 2) high haplotype and nucleotide diversity should characterize species structure, being the signature of localized refugia when associated to high geographic structuring and to lack of population expansion (Mosblech et al., 2011); 3) deep splits are expected, since the lack of contemporary high gene flow would not have erased the signature of ancient events.

4.3 MATERIALS AND METHODS

Sample collection

The samples of *Apolochus neapolitanus* and *A. picadurus* were collected in *P. oceanica* meadows along the coasts of Central Mediterranean Sea from 9 localities distributed in Central and Southern Tyrrhenian Sea, Strait of Sicily and Adriatic Sea (Fig. 1, Table 1). According to the sampling techniques reported in Camisa et al. (2017), an air-lift sampler (500-µm mesh size) was used while diving to collect the vagile fauna from sampling quadrants. Sampling was always carried out at summer (between July 2015 and September 2017) and at the same depth (15-20 m), since both season and depth may influence the abundance of amphipod species associated with the meadows (Camisa et al., 2017; Mazzella et al., 1992). The samples were preserved in 70% ethanol until sorted out in laboratory using a binocular. The taxonomic identification at the species level of the sampled individuals was based on the handbooks by Ruffo (1982, 1989, 1993, 1998), updating the nomenclature according to the World Register of Marine Species. A blind subset of the *Apolochus* individuals identified was sent to an independent expert for assignment validation.

DNA extraction and PCR amplification

Total genomic DNA was extracted either from the whole individuals or from small amount of tissue using different techniques: on whole individuals we used the Universal DNA extraction Kit developed by Zymo Research following manufacturer's instructions, while using the HOT/SHOT protocol we were able to obtain appropriate amounts of DNA from pereopods (Montero-Pau et al. 2008). Polymerase chain reaction (PCR) was carried out to amplify fragments of two molecular markers: the mitochondrial Cytochrome Oxidase I (COI) and the nuclear Histone 3 (H3). Universal primers for marine invertebrates LCO1490/HCO2198 were used for COI amplification (Folmer *et al.* 1994) according to the protocols described in Lobo et al. (2016) and Marusso et al. (2013), with minor modifications. PCR reactions were performed in 25 µl of 10x reaction buffer, 3 µl of MgCl₂, 0.5 µl of dNTPs, 5 pmol/l of each primer and 1 unit of *Taq* DNA polymerase (Promega). PCR thermal condition for COI were conducted with an initial denaturation cycle of 1 min, followed by 5 cycle: 94°C denaturation for 60 seconds, 48°C/90 seconds, 72°C/60 seconds and 35 cycles: 94°C/30 seconds, 52°C/90 seconds, 72°C/60 seconds and a final extension at 72°C for 10 minutes. To amplify H3 gene, H3F and H3R primers from Colgan et al. (2004) were used, slightly modifying the PCR protocol from Hou et al. (2007). PCR thermal condition were conducted with an initial denaturation of 94°C/60 seconds followed by 40 cycles of 94°C/30s, 48°C/45 seconds, 72°C/60 seconds and 8 minutes final extension at 72°C. PCR products were visualized in a standard 1.2% agarose gel electrophoresis and then sent to Macrogen Inc (<https://dna.macrogen.com>) for purification and sequencing.

Sequences alignment and Phylogenetic analysis

Electropherograms were checked by eye using 4Peaks (Nucleobytes BV) and aligned using the software MEGA v7.0.7 with the Clustal algorithm (Kumar et al., 2016). Preliminary phylogenetic analyses incorporating COI sequences obtained from this study and the only other COI sequence available (*A. neapolitanus* BCASB012-16, Atlantic, North Spain, retrieved from BOLD system) were carried out to find the best outgroup, *Talorchestia martensii* (GenBank MG65588). Prior to infer phylogenetic reconstruction we selected the best evolution model using J-Model test 2.1.3 under the Akaike Information Criterion, finding out the “transversion model” with gamma distribution: TVM+G for COI; and “3-parameter model” with gamma distribution: TPM1+G for H3 (Darriba et al., 2012). We performed phylogenetic reconstruction based on two different methodologies:

maximum likelihood (ML) and Bayesian Inference (BI). BI trees were reconstructed using MrBayes v2 with haplotypes including two runs of ten million generations (Drummond et al., 2006). Trees were sampled every 1000 generations using four Markov chains and the default heating values. The first 25% of sampled trees were discarded as burn-in. Convergence was assessed by the standard deviation of split frequencies (>0.01) and by examining the trace plot in TRACER 1.6 (Rambaut et al., 2014). The burn-in was fixed to 10% and the remaining trees were used to construct the 50% majority rule consensus tree. ML tree was performed using IQTREE (Trifinopoulos et al., 2016). Haplotype networks for COI mitochondrial marker were estimated using a Median Joining algorithm (Bandelt et al., 1999) in NETWORK 5.0.0.3 (<http://www.fluxus-engineering.com>).

Gene trees for H3 nuclear marker were converted to haplotype networks using HAPLOTYPE VIEWER (Guindon et al., 2004).

Cryptic diversity and MOTUs delimitation

To delimit the presence of cryptic species in our samples we performed two different analyses on COI dataset. First we run the analysis in ABGD (Automatic Barcode Gap Discover) server (<http://www.wabi.snv.jussieu.fr/public/abgd/abgdweb.html>), which recognizes molecular taxonomic units (MOTUs) by identifying inter-species gaps in the pairwise distance distribution (Puillandre et al. 2012). The second method used was GMYC (Generalized Mixed Yule Coalescent), which measures the transition from intra- to interspecific branching patterns to identify species boundaries (Pons et al., 2006; Reid and Carstens 2012). Prior to the analysis, we removed the outgroup and then used unique haplotypes to obtain an ultrametric consensus tree as starting point for the GMYC model. To this end, we used the software BEAST v2 (Bourckaert et al., 2014) to run two independent runs of ten million generations each under a Yule model and a constant clock, assuming that mutation accumulates at a constant rate of evolution throughout the tree. This model works well when applied to related species, especially when a single marker is involved (Fujisawa and Barraclough, 2013). The ultrametric tree was checked by visualizing the posterior with Tracer and by verifying that the values of the ESS (Estimated Sample Size) were >200 ; we then summarized the tree with TREEANNOTATOR. Finally, to estimate the number of MOTUs included in the tree we imported the data and the tree in R 3.3.2 (R Core Development Team 2014) and carried out GMYC using the

package “splits” with the unique threshold method (Species Limits by Threshold Statistics; Ezard et al., 2009).

Population genetic structure, molecular diversity and demographic analysis

To investigate the population genetic structure and to further support the number of MOTUs identified, we run a spatial genetic mixture analysis for clustering DNA sequence data in BAPS: Bayesian Analysis of Population Structure v. 6.0 (Corander et al., 2008) using COI dataset. The analysis was carried out assuming a priori a maximum number of populations with K=10, K=20 and K=30 for three replicates in each of the three runs. The K value with the highest posterior probability was chosen as the best partitioning.

Molecular diversity indices (H, number of haplotypes; h, haplotype diversity; π nucleotide diversity) were estimated for each MOTU recovered by previous analyses using DNAsp v.6 (Rozas et al. 2007). Pairwise F_{ST} values, K2P and p distances were calculated using MEGA v.7 (Kumar et al., 2016). Mismatch distribution analysis and neutrality test for each MOTU were performed in Arlequin 3.5.1.3 (Excoffier and Lischer, 2010) in order to test for historical demographic or spatial expansion by Tajima’s D test and Fu’s F_s (1000 replicates each).

All the trees obtained were edited in FigTree v1.4.3 (Rambaut, 2018).

Divergence time estimation

Time calibration was carried out by reconstructing a phylogeny in BEAST v2 (Bourckaert et al., 2014) on COI dataset. The scarcity of paleontological fossils for amphipods prevented any calibration, so the substitution rate of 0.0115 per site per million year was used to estimate divergence times. This rate has been applied in recent studies on amphipods belonging to the genera *Talorchestia* and *Gammarus* supporting plausible scenarios (Liu et al., 2018; Hupalo et al. 2019). The run was performed under an uncorrelated lognormal relaxed molecular clock (Brower, 1994). We applied a Yule tree prior with birth rate variation following a lognormal distribution with a mean of 1 and a standard deviation of 0.15. We performed two runs of 20 million generations sampling every 1000th and discarding the first 25% as burn-in. Convergence to the stationary distribution and node-height estimates were checked by plotting the marginal posterior probabilities versus the generation state using TRACER, together with ESS values (all >200).

The posterior probability density of the combined tree and log files was summarized in a maximum clade credibility tree with TREEANNOTATOR (Bouckaert et al. 2014). The posterior probabilities of the clades and the mean and 95% highest posterior density interval of divergence times were visualized in FigTree v1.4.3 (Rambaut, 2018).

4.4 RESULTS

We obtained sequences of mitochondrial COI (671 bp) and of nuclear H3 (341bp) from 101 individuals belonging to two different morphospecies. COI showed 310 polymorphic sites (276 parsimony informative), resulting in 83 haplotypes while H3 had 128 polymorphic sites (127 parsimony informative) and 46 haplotypes (Table 1). All unique sequences have been deposited in GenBank (accessions added upon acceptance). Nucleotide percentages were: T 34.7%, C 18.5%, A 22.5% and G 24.1% in COI; T 22.6%, C 30.7%, A 23.5% and G 23.1% in H3.

COI diversity and MOTUs identification

We retrieved 68 haplotypes from the 78 specimens of *A. neapolitanus* collected from 7 localities (Table 2). All the recovered haplotypes were private (i.e., found in a single population), except a single haplotype (H9) shared between two Tyrrhenian localities. A similar pattern was recovered for *A. picadurus*, since all the 15 haplotypes observed in the 23 specimens analysed were found in a single locality.

The genetic differentiation recorded between Mediterranean *A. neapolitanus* and *A. picadurus* was high, with an average K2P distance of 0.288. Intraspecific values were also high, being 0.157 and 0.143, respectively, and so suggesting that cryptic diversity is hidden within these species.

This hypothesis was confirmed by the congruent results obtained using ABGD and GMYC methods to recover MOTUs: 3 MOTUs were found within each *Apolochus* morphospecies, with a fourth in *A. neapolitanus* represented by the Atlantic specimen (Fig. 2). Within *A. neapolitanus*, a first MOTU (called N1) included specimens from Western Sicily and Central Tyrrhenian Sea, the second (called N2) grouped all and only the specimens from Eastern Sicily (MZ), while the third MOTU (called N3) had a wider distribution including Tyrrhenian Sea (GIAN, MOR), Western Sicily (MAR) and Adriatic Sea (OT). GMYC recognized one more MOTU in N3 formed by some samples

of the Tyrrhenian Sea (MOR). The only Atlantic sequence of *A. neapolitanus* constituted a fourth MOTU (N4). The three *A. picadurus* MOTUs included respectively individuals from Adriatic (OT) and Southern Tyrrhenian (BR) Seas (in the MOTU called P1), individuals from Central Tyrrhenian Sea (GIAN, MOR) in P2 and individuals from Southern Tyrrhenian Sea (SCI, SCA) in P3 (Fig.2). MOTUs N3 and P1 were present in all the three seas studied (Tyrrhenian, Strait of Sicily, Adriatic); the more restricted distribution observed for the other MOTUs could reflect the real range of the species, but maybe also due to sampling artefacts, with the possible exception of *A. neapolitanus* N2, which is the only MOTU recovered in the Sicily Channel and so likely representing an endemism.

BAPS analysis recovered 7 as the best group membership solution with a posterior probability of 1.00 and these seven Mediterranean groups were mainly coincident with those found with MOTU delimitation (Fig.2). The analysis confirmed that the identified groups were not geographically separated, since MOTUs belonging either to the same or to different morphospecies, could co-occur in the same locality (Fig. 1).

Both Bayesian and ML analyses recovered the same topology, clearly dividing *A. neapolitanus* and *A. picadurus* clades, further subdivided in 7 sub-clades corresponding to MOTUs identified by ABGD (Fig. 2). Given the similarity of results in grouping the samples, we decided to retain the subdivision in 7 clades/MOTUs shown in Fig. 2 to perform further analyses. The same analyses (BA and ML) were carried out on H3 sequences, showing less supported results. The network analysis subsequently reported showed an overall agreement between the two markers, although some mitonuclear discordance emerged.

Genetic population structure and mitonuclear discordance

The genetic differentiation observed at COI within the two morphospecies was high although comparable to that recorded within other amphipod species complexes (Hupalo et al., 2019): *A. neapolitanus* MOTUs showed K2P between 0.102 and 0.271 and, similarly, *A. picadurus* MOTUs diverged between 0.127 and 0.276 (Tables 3, 4). On the contrary, the distances recorded within each MOTU ranged between 0.001 (P2) and 0.038 (N3), one or two orders of magnitude lower than the interclade distance. The higher values of haplotype and nucleotide diversity were shown by *A. neapolitanus*; the clade P3 of *A. picadurus* showed $h = 1$ but over a small number of specimens (Table 3).

The network analysis carried out on COI sequences recovered seven highly differentiated

groups, if we consider the Atlantic specimen, corresponding to the MOTUs previously identified (Fig. 3a). When the localities where each haplotype was found were shown on the network, it emerged that the haplotypes of *A. neapolitanus* were never shared with the exception of H9, which was found both in Central Tyrrhenian (GIAN) and Western Sicily (MAR) (Fig. 3b). None of the clades showed a star-like structure and the haplotypes were highly differentiated within each clade. In N1 a group of close haplotypes was shared between three Tyrrhenian localities (GIAN, MAR, MDV) with two groups of highly differentiated haplotypes distributed mainly (although not exclusively) in MAR and MDV (Fig. 3b). N2 showed 18 highly differentiated haplotypes recovered in a single locality (MZ) and never occurred in the other Sicilian samples (Fig. 3b). N3 haplotypes showed a clear geographic structuring, with Adriatic, Western Sicily, Sicily Strait and Central Tyrrhenian as distinct subclusters, with the only exception of GIAN haplotypes, which clustered within the Sicilian group (MAR) (Fig. 3c). In *A. picadurus*, the clade P1 is recovered in southern Tyrrhenian (BR) and Adriatic Sea (OT), each one hosting well differentiated groups of haplotypes, while the clade P2 was found in central Tyrrhenian only and P3 was localized in southern Tyrrhenian Sea (Fig. 3d).

Mean genetic distances values for H3 between MOTUs were not high as the values recorded for COI marker. *A. neapolitanus* showed K2P between 0.013 and 0.049, while *A. picadurus* divergence ranged between 0.038 and 0.124. Intragroups distances did not show the same pattern found at COI, where we obtained distances one or two orders of magnitude lower than the interclade distance (Table 3).

In general, distances recorded within and between MOTUs obtained with H3 marker well discriminated the two major groups corresponding to morphospecies: *A. picadurus* and *A. neapolitanus*, while it showed poor resolution in discriminating the MOTUs recognized using COI.

The network analysis carried out on H3 gene sequences showed a pattern similar to that of COI dataset in evidencing cryptic diversity within the two morphospecies, as shown by the pattern of mean genetic distances. However, some haplotypes were linked to different groups with respect to the assignment carried out according to both morphology and COI. The network analysis in Fig. 4a showed the clades reported with the colours corresponding to COI MOTUs subdivision, to highlight some cyto-nuclear discordance in a few specimens. Four haplotypes recovered from three specimens of Adriatic Sea (OT) were classified as *A. neapolitanus* according to both morphology and COI but were grouped as *A. picadurus* according to H3 (H17, H18, H19, H22). Another Adriatic haplotype

(H34 from OT) was classified as *A. picadurus* based on both morphology and COI but was clustered within *A. neapolitanus* according to H3 and the same happened to 4 haplotypes from Southern Tyrrhenian Sea (H42, H44, H45, H46 from SCI). Only one of the specimens carrying these haplotypes was heterozygote (*A. neapolitanus* from OT, bearing H17/H18) and none of the specimens proved to be a F1 hybrid (i.e. showed the co-presence of nuclear haplotypes belonging to different MOTUs). The cyto-nuclear discordance observed could therefore be due to incomplete lineage sorting at H3 marker more than to hybridization, as reported also by different authors (Ji et al., 2016; Wood et al., 2018; Schnurr et al., 2018) stating that H3 marker could be affected by an incomplete lineage sorting and consequently it could not provide a congruent and plausible phylogenetic reconstruction if compared with other genetic markers. When the network evidenced the localities where each haplotype was found, in *A. neapolitanus* two haplotypes resulted widespread across the whole central Mediterranean Sea: H1 and H4 were found in Central and Southern Tyrrhenian Sea and in Adriatic as well. In *A. picadurus* the network showed that each locality hosts different and private haplotypes.

Divergence times and historical demography

The time calibrated phylogeny showed that the diversification within each morphospecies started during the Terminal Tethyan Event (TTE, 18-12 Mya, CIT), dating back at 17 Mya in *A. picadurus*, and at 12 Mya in *A. neapolitanus*. In *A. neapolitanus* (Fig. 5) MOTU N1 was the first to diverge (12 Mya), followed by the split of the Atlantic MOTU N4 about 11 Mya. The further divergence between N1 and N2 took place more recently, around 4.6 Mya. The intra-clade divergence occurred during Pleistocene, in the last 2 Mys. In *A. picadurus* (Fig. 6) MOTU P3 diverged about 17 Mya, while the split between MOTUs P1 and P2 occurred around 6.7 Mya. Intraclade divergence occurred mainly during the last 400-100,000 years, with the exception of the two P1 sub-clades, which diverged around 2 Mya.

None of the MOTUs identified showed any signature of demographic expansion according to all the values recorded (Table 2).

4.5 DISCUSSION

Cryptic diversity and highly fragmented population structure

Amphipods are a speciose group of Peracarida, with over 10,000 species described to date, inhabiting a variety of terrestrial, freshwater and marine habitats (Horton et al., 2018). Marine amphipods have been largely neglected in phylogeographic approaches till recent years, when an increasing number of studies showed a high level of cryptic diversity and genetic structuring all around the world. Antarctic amphipods have been proposed as an example of Species Flock (i.e. a monophyletic group showing high speciosity, ecological diversity and dominance) characterized by a high rate of cryptic speciation (Chenuil et al., 2018). The presence of cryptic, highly divergent lineages has been evidenced in species inhabiting naturally fragmented habitats like Atlantic and Mediterranean brackish waters (*Gammarus aequicauda* and *G. insensibilis*; Hupalo et al., 2019) or Japanese sandy beaches (*Haustorioides japonicus*; Takada et al., 2018). Here, the differentiation could be explained as a consequence of habitat discontinuity, but high intraspecific diversity has been evidenced also in marine benthic amphipods studied from Antarctica to Caribbean to North Sea (Baird et al., 2011; Richard et al., 2012; Iacofano and Lo Brutto, 2017; Beermann et al., 2018). Even in the pelagic environment cryptic diversity was revealed in the free swimming *Themisto* (Tempestini et al., 2017).

The results obtained in this study fall within this frame, by evidencing high cryptic diversity in the two morphospecies *A. neapolitanus* and *A. picadurus*. Four and three MOTUs respectively have been identified, showing differentiations between 10 – 28%, so comparable or higher than those found in other amphipod species complexes (Tempestini et al., 2018).

The geographic distribution of these putative species showed that they have been able to cross relevant Mediterranean barriers and to distribute in different seas over the time. This is the case of *A. neapolitanus* N3, which has been found in central and southern Tyrrhenian Sea, western Sicily and Adriatic Sea. Similarly, *A. picadurus* P1 was recovered in both southern Tyrrhenian and Adriatic samples. This despite the Adriatic Sea is a semi-enclosed basin showing a moderate water mass exchange with the Ionian and southern Mediterranean seas through the Albanian side of the Strait of Otranto (Orlic et al., 1992). The effectiveness of the Otranto Strait on benthic amphipods has also been recently demonstrated by biogeographic studies of amphipods assemblages associated to

Posidonia oceanica meadows: Adriatic communities are not only less rich in species but are also characterized by the presence of cosmopolitan species, better able to cross major barriers (Bellisario et al., 2019). However, the remarkable level of genetic structuring here observed both between and within the different biogeographic areas suggests that even within single MOTUs the levels of ongoing gene flow are low or absent, as witnessed by the lack of shared haplotypes among samples. This observation is only apparently in contrast with the effectiveness of the oceanographic barriers considered, being in substantial agreement with the biological and ecological features of the studied species. The lack of a larval pelagic stage in amphipods, that are brooding species, prevent their massive dispersal through marine currents, so making them fully dependent from rafting or other forms of adult passive dispersal. As a consequence, both the colonization of new areas and the gene flow should occur through rare long distance dispersal events. The signature of such extremely low vagility is known to be a pattern of genetically highly fragmented populations, with comparable differentiation among populations located both on the same and on the opposite sides of geographic fronts, as here recorded within each *Apolochus* species complex (Pascual et al., 2017).

Deep splits in Apolochus species complex

According to their low vagility and subsequent models of gene flow *Apolochus*, as other benthic amphipods, should be able to retain the signature of ancient biogeographic events, not overwritten by recent admixture episodes (Provant and Bennett, 2008). Correspondingly, the diversification within *Apolochus* morphospecies has been found to be quite ancient, and seems to be strictly linked to the Terminal Tethyan Event (TTE, 18-12 Mya, CIT) either at its early (in *A. picadurus*, around 17 Mya) or final stage (in *A. neapolitanus*, around 12 Mya). Accordingly, no population/clade showed signs of recent expansion. A deep split of basal clades in Mediterranean amphipods is finding increasing support while phylogeographic studies accumulate. Ancient lineages stemming around 15-13 Mya have been recently evidenced in *Talorchestia* (Liu et al., 2018) and in *Gammarus aequicauda* complex the divergence could be even more ancient, dating back to early Miocene (Hupalo et al., 2019).

The four putative species identified in *A. neapolitanus* emerged at different times, between 12 to 5 Mya, according to some of the major paleogeographic events of the Mediterranean history. The divergence of Atlantic N4 occurred in Late Miocene about 10.8 Mya, quite close to the basal split of N3. Both these events could be related to the complex orogenic changes due to the progressively

accelerating collision of African and Euroasiatic plates, which not only closed the connections toward east (TTE), but also triggered the subdivision of the central Parathetys into many small sub-basins only temporarily interconnected (Bianchi et al., 2012; Steininger and Rögl, 1984). In agreement with our findings, this great complexity of the Mediterranean Sea in late Tortonian has been recently associated to the emergence of the main lineages observed in another poorly vagile species, the gastropod *Dendropoma petreum* (Calvo et al., 2015). N1 and N2 lineages emerged at the end of the Messinian Salinity Crisis (MSC, 5.33 Mya), when the Pliocene flooding ended one of the most relevant events in the Mediterranean history (Lourens et al., 1996). Further divergence within each putative species of *A. neapolitanus* complex occurred during the Plio-Pleistocene (since 3.7 My), in correspondence with the occurrence of glacial and inter-glacial cycles and corresponding eustatic sea-level fluctuations. The same events shaped *A. picadurus* history, with P3 diverging since the beginning of TTE and the split between P1 and P2 occurring around 6 Mya, so at the beginning of MSC. This is considered one of the most dramatic periods in the history of the Mediterranean Sea, triggered by the vertical movements in the Betic and Rif areas during late Miocene. These affected the basin's hydrography and, together with the constriction of the portals between Atlantic and Mediterranean, led to the isolation of the basin through the closure of the Strait of Gibraltar and brought the Mediterranean into the "Lago-Mare" stage, when brackish to hyperaline lakes occupied the largely dried basin (Krijgsman et al., 1999). Although the MSC has been claimed to cause the nearly total extinction of marine species, allowing the survival of euryhaline or brackish species alone, there is increasing evidence that the desiccation process of MSC did not occur simultaneously across the whole basin and that the process underwent cyclical alternation, so warranting the presence of marine refuges throughout the Messinian Age (Mejier and Krijgsman, 2005). Accordingly, there is increasing evidence of the survival of paleo-endemisms within marine species, including many crustaceans (Schubart et al., 2001; Sotelo et al., 2009; Reuschel et al., 2010). The further divergence in the two P1 sub-clades dates back to late Pliocene-early Pleistocene while the differentiation of present populations is more recent, between 400-100,000 years ago, so linked to the alternation of pleistocenic ice ages.

4.6 CONCLUSIONS

The finding of the high, cryptic diversity in the two studied species *A. neapolitanus* and *A. picadurus* across a relatively circumscribed area as the central Mediterranean Sea confirms the growing bulk of data highlighting that the real levels of biodiversity may be overlooked in marine taxa if not deeply studied using molecular approaches. This could seem surprising in organisms like benthic amphipods that are considered as key components of coastal communities and play a relevant role in transferring energy to upper trophic levels (Michel et al., 2015; Bellisario et al., 2016). However, the occurrence of cryptic speciation associated with morphological stasis has been repeatedly observed in amphipods, explaining the difficulties in detecting highly divergent lineages using traditional approaches (Bickford et al., 2007; Richards et al., 2012).

Our results also provide insights into the usefulness of studying poorly vagile organisms, which are present also in the marine habitats, to assess the biogeographic history of the Mediterranean Sea. Till recent years, the most studied species were characterized by a high vagility, since the main focus was on the phylogeographic break between Atlantic and Mediterranean Sea (Cimmaruta et al., 2005; Patarnello et al., 2007). Only recently the analysis of the biogeographic barriers within the Mediterranean Sea has risen to popularity, since they are increasingly proved to have been effective in determining genetic structuring and lineage distribution within species, together with their life history traits (Villamor et al., 2014; Pascual et al., 2017). This happened also because the study of organisms with limited dispersal ability allowed avoiding the loss of the signature of ancient events because of the superimposition of contemporary high gene flow (Provant and Bennett, 2008). Our results demonstrated that amphipods are among these organisms and that their study is contributing to change the perception of the Mediterranean biodiversity as having a recent, post-Messinian origin, toward a more ancient derivation thanks to species survival along MSC (see for example Liu et al., 2018; Hupalo et al., 2019).

4.7 TABLES AND FIGURES

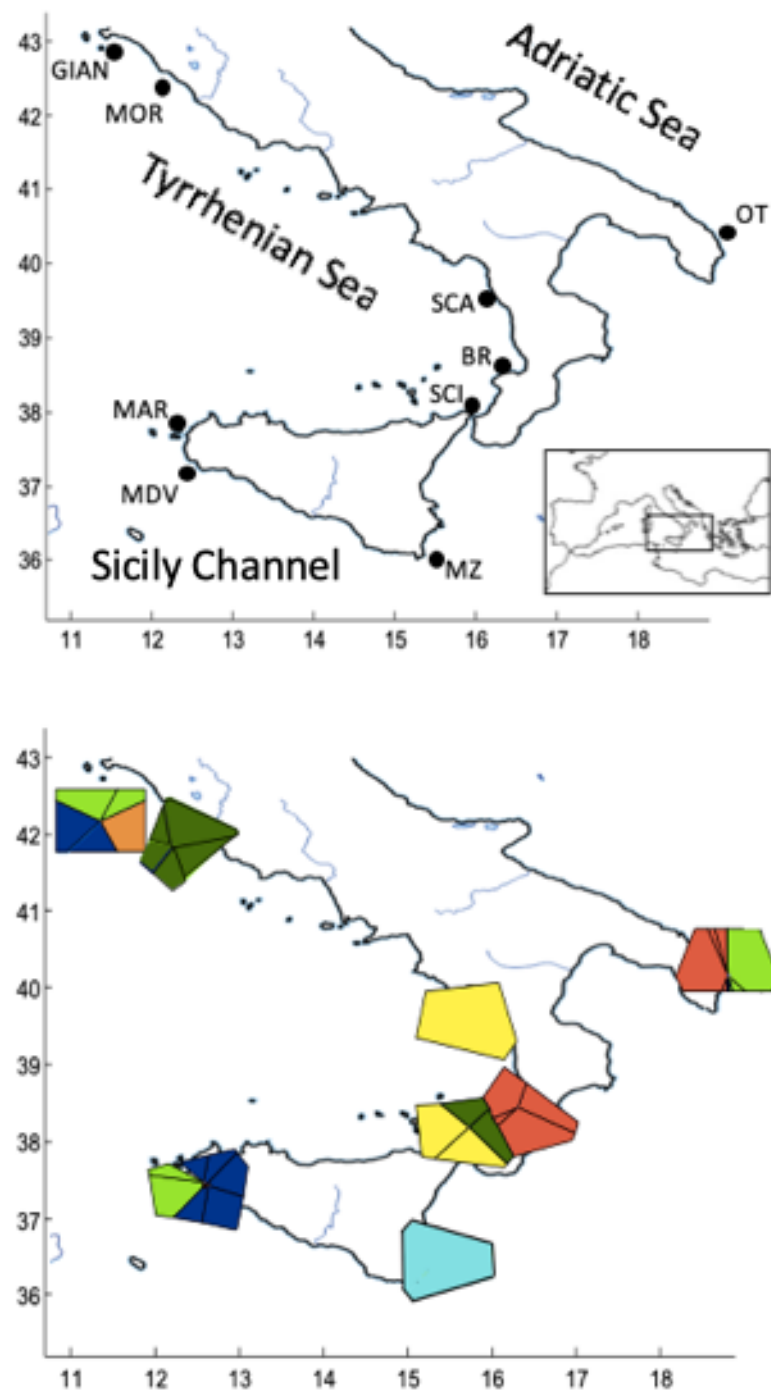


Figure 1 – Map showing the sampling sites and the results of the spatial genetic mixture analysis using BAPS. Different colours correspond to the different clusters recovered.

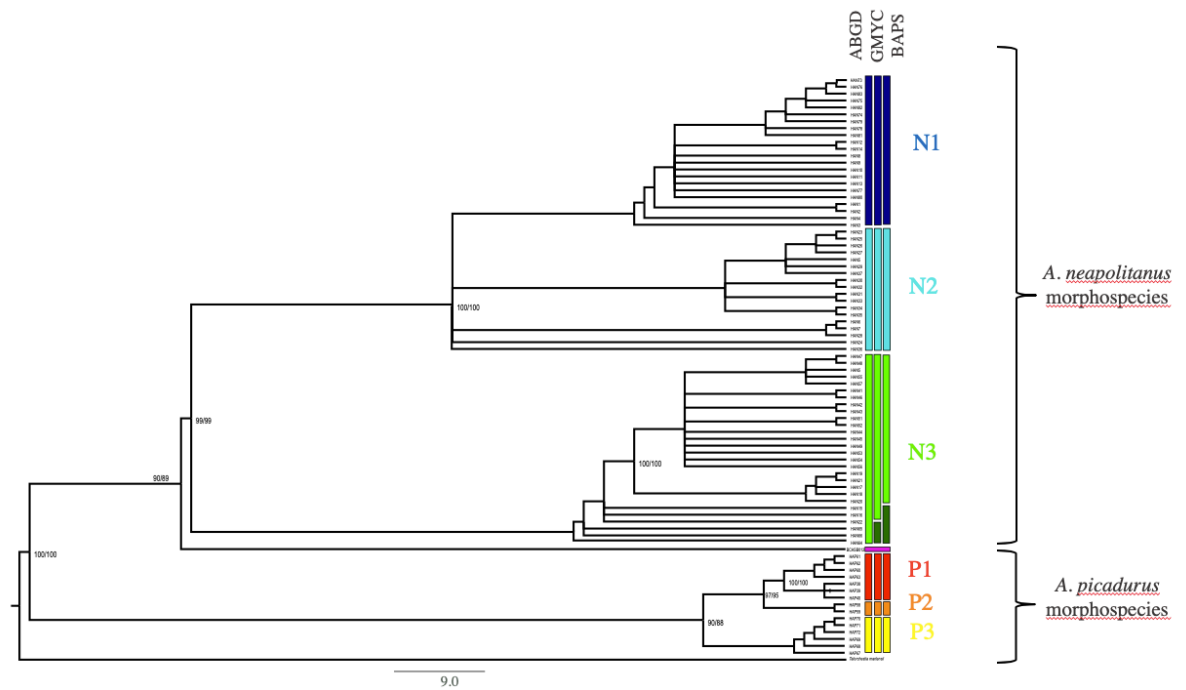


Figure 2 – Bayesian tree derived from mitochondrial COI gene sequences of *Apolochus neapolitanus* and *A. picadurus*. Support values are reported at nodes (Bayesian posterior probability ≥ 80 / Maximum likelihood bootstrap percentage ≥ 80). Colour bars indicate the MOTUs recovered according to ABGD, GMYC and BAPS in *A. neapolitanus* (dark blue N1, light blue N2, green N3, pink N4) and *A. picadurus* (red P1, orange P2, yellow P3).

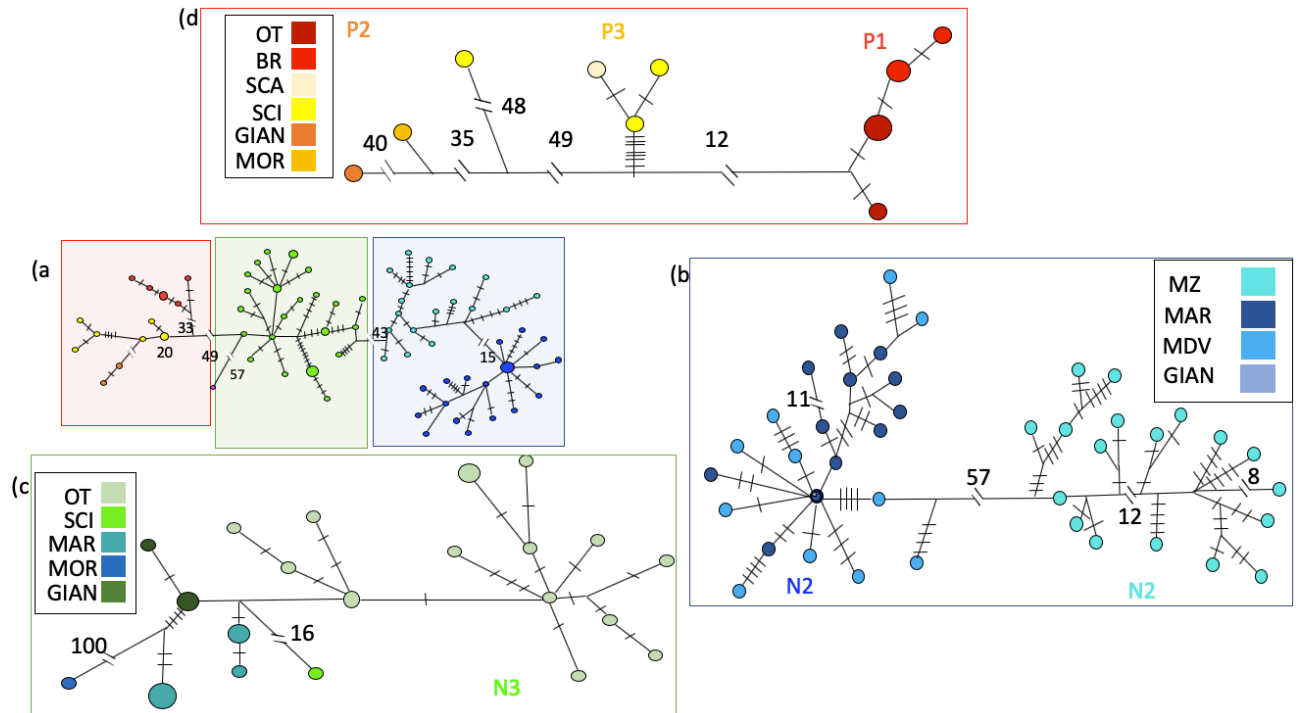


Figure 3 – COI haplotype network of *Apolochus neapolitanus* and *A. picadurus* species complex, with branch length proportional to mutational steps and circle size proportional to the frequency of each haplotype. In (a) is represented an overview of the MOTUs identified within *A. neapolitanus* (dark blue N1, light blue N2, green N3, pink N4) and *A. picadurus* (red P1, orange P2, yellow P3). In (b), (c), (d) the different colours correspond to the localities where each haplotype was found for MOTUs N1 and N2 (b), MOTU N3 (c) and MOTUs P1, P2, P3 (d).

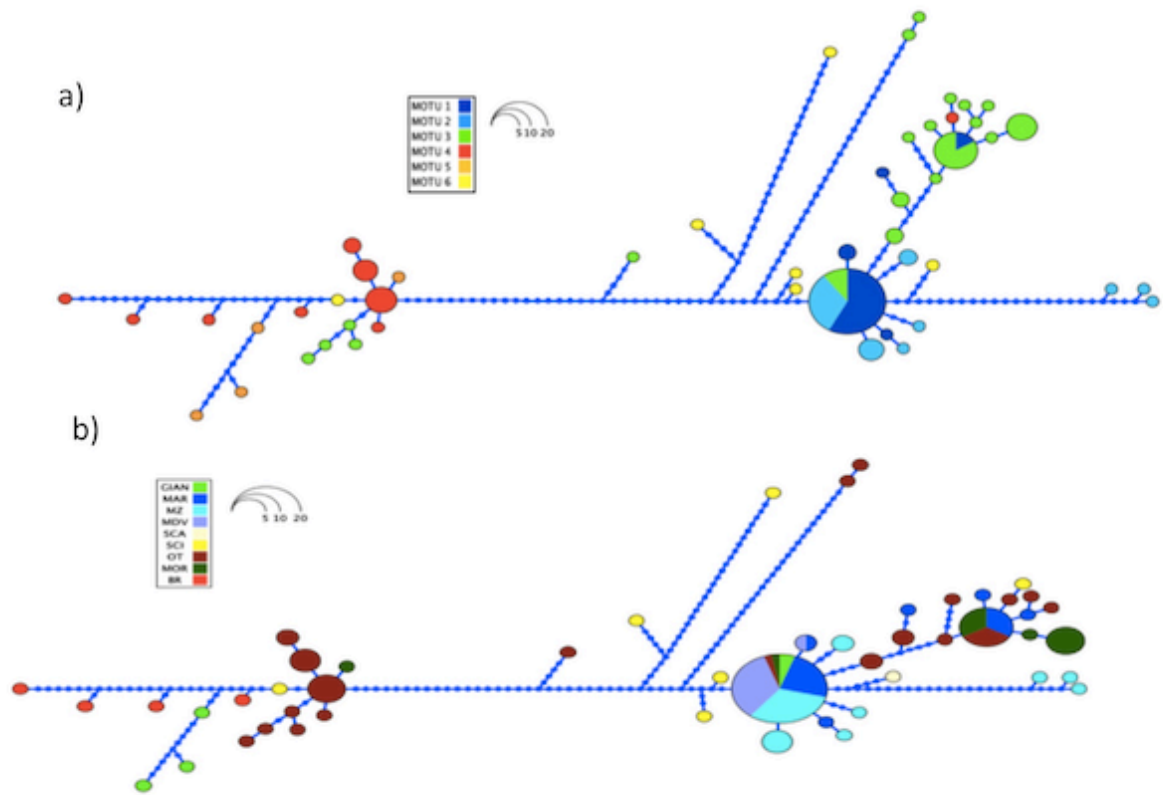


Figure 4 – HISTONE 3 gene haplotype network of *Apolochus neapolitanus* and *A. picadurus* species complex, with branch length proportional to mutational steps and circle size proportional to the frequency of each haplotype. In (a) is represented an overview of the MOTUs identified within *A. neapolitanus* (dark blue N1, light blue N2, green N3, pink N4) and *A. picadurus* (red P1, orange P2, yellow P3) with colours according to COI data (see the text for further explanations); in (b) the different colours correspond to the localities where each haplotype was found.

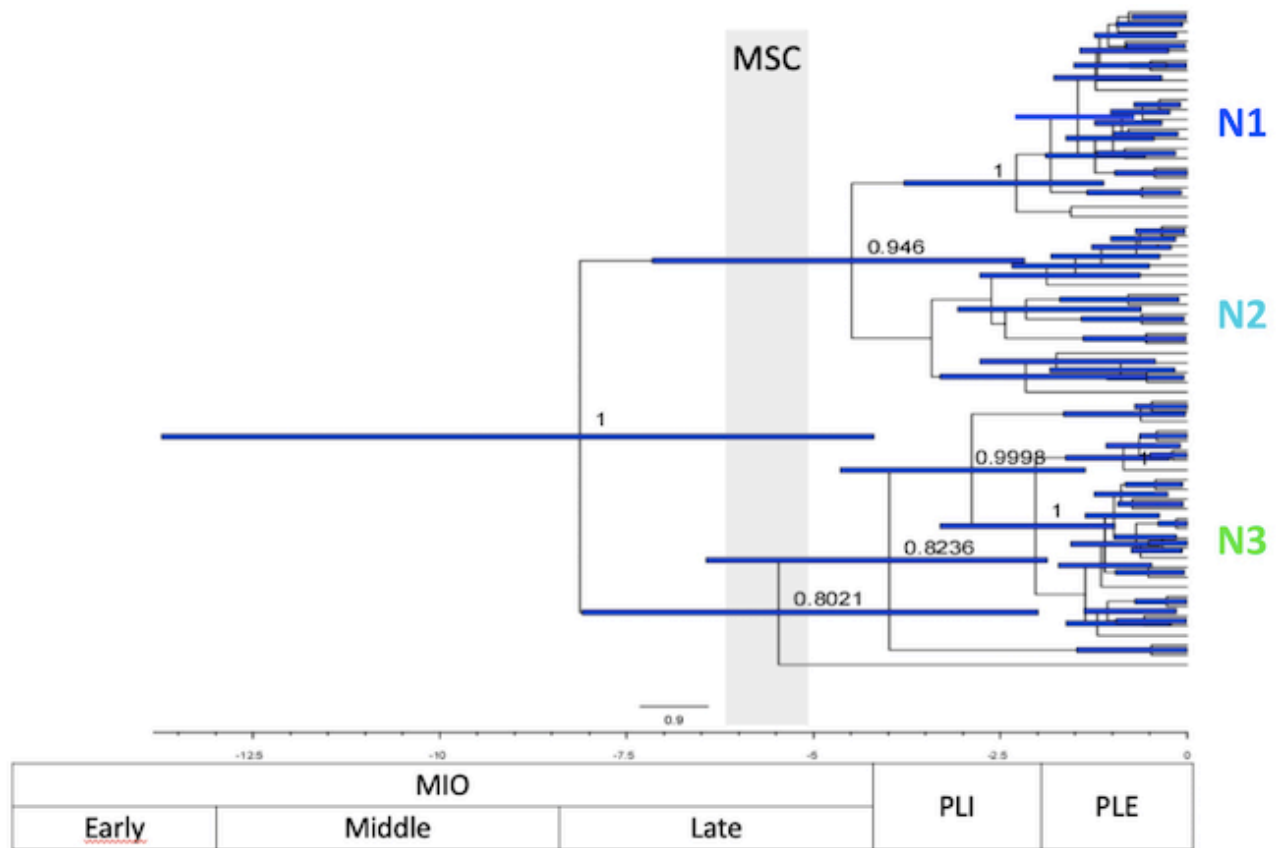


Figure 5 – Maximum clade credibility, time-calibrated Bayesian reconstruction of *Apolochus neapolitanus* species complex based on COI data. Support values are reported at nodes (Bayesian posterior probability ≥ 80) and node bars represent 95% HPD.

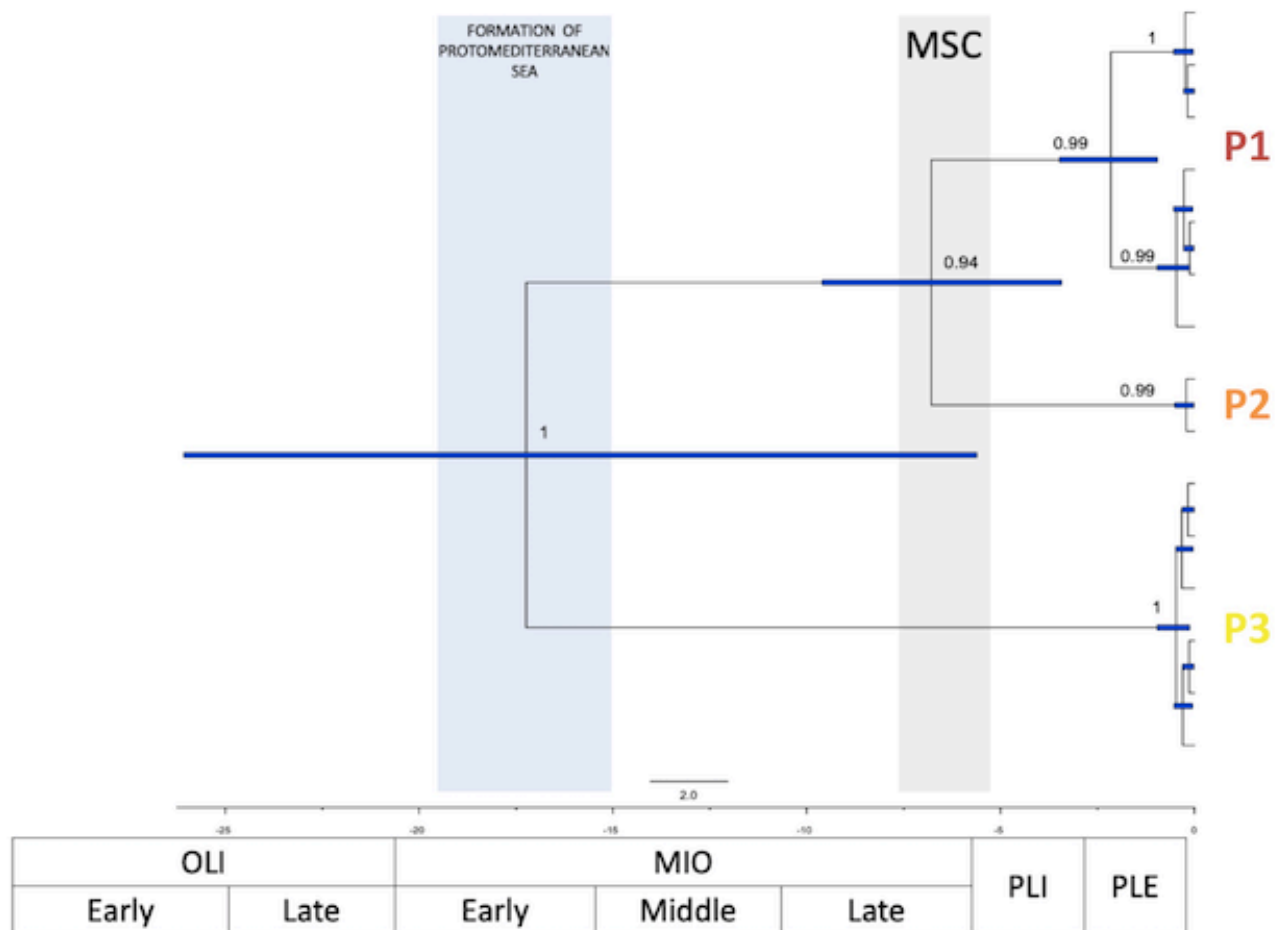


Figure 6 – Maximum clade credibility, time-calibrated Bayesian reconstruction of *Apolochus picadurus* species complex based on COI data. Support values are reported at nodes (Bayesian posterior probability ≥ 80) and node bars represent 95% HPD.

Table 1. Sampling sites with their and code and coordinates.

Sampling site	Geographic area	Code	Latitude	Longitude
Giannutri Island	Tyrreahnian Sea	GIAN	42 15415	11 06284
Punta Murelle	Tyrrhenian Sea	MOR	42 19419	11 30247
Marsala	Sicily Strait	MAR	37 44135	12 28025
Mazara del Vallo	Sicily Strait	MDV	37 39324	12 33374
Marzamemi	Sicily Strait	MZ	36 44490	15 06579
Briatico	Tyrrhenian Sea	BR	38 43592	16 01552
Scalea	Tyrrhenian Sea	SCA	39 46588	15 46589
Scilla	Tyrrhenian Sea	SCI	38 15194	15 42487
Otranto	Adriatic Sea	OT	42 15415	11 06284

Table 2. Number of specimens analysed per MOTU with genetic diversity parameters (H, Hd, pi), neutrality tests (Tajima's D, Fu's Fs) and mismatch analysis for demographic and spatial expansion.

	N	H	Hd	pi	Tajima test	Fu's FS test	demographic expansion		spatial expansion	
							SSD	RI	SSD	RI
N 1	26	22	0,985	0,0167	-1.595 <i>0.036</i>	-7.875 <i>0.004</i>	0.0055 <i>0.96</i>	0.0099 <i>0.910</i>	0.0095 <i>0.8400</i>	0.00997 <i>0.9300</i>
N 2	19	18	0,994	0,0225	- 0.7621 <i>0.2180</i>	-5.836 <i>0.004</i>	0.0307 <i>0.000</i>	0.0292 <i>0.2800</i>	0.0141 <i>0.1200</i>	0.0292 <i>0.1600</i>
N 3	33	28	0, 983	0, 0363	- 0.0382 <i>0.5460</i>	-5.224 <i>0.0480</i>	0.0131 <i>0.3300</i>	0.0068 <i>0.8100</i>	0.0170 <i>0.2500</i>	0.0068 <i>0.8300</i>
P 4	13	7	0, 795	0, 0209	1. 2413 <i>0.9220</i>	3.474 <i>0.9320</i>	0.1274 <i>0.0900</i>	0.1407 <i>0.3500</i>	0.0873 <i>0.1300</i>	0.1407 <i>0.500</i>
P 5	4	2	0, 500	0, 0014	- 0.7099 <i>0.2910</i>	1.098 <i>0.6190</i>	0.1892 <i>0.51000</i>	0.7500 <i>0.7800</i>	0.1364 <i>0.1500</i>	0.7500 <i>0.9200</i>
P 6	6	6	1, 000	0, 0054	- 0.4154 <i>0.3790</i>	-2.813 <i>0.0190</i>	0.0138 <i>0.8200</i>	0.0711 <i>0.7700</i>	0.0141 <i>0.8500</i>	0.0711 <i>0.7500</i>

Table 3. Mean genetic distances (K2P) between MOTUs; H3 above the diagonal and COI below the diagonal.

	N 1	N 2	N 3	P 1	P 2	P 3	N 4 (Atlantic O.)
N 1	*	0.013	0.039	0.119	0.142	0.053	-
N 2	0.102	*	0.049	0.127	0.149	0.061	-
N 3	0.271	0.254	*	0.114	0.136	0.074	-
P 1	0.289	0.299	0.259	*	0.038	0.105	-
P 2	0.286	0.281	0.278	0.127	*	0.124	-
P 3	0.321	0.334	0.295	0.276	0.260	*	-
N 4	0.287	0.279	0.251	0.231	0.235	0.313	*

Table 4. Mean genetic distances within MOTUs.

	P- DISTANCE (COI)	K2P (COI)	P- DISTANCE (H3)	K2P (H3)
N 1	0.017	0.017	0.006	0.006
N 2	0.023	0.023	0.019	0.019
N 3	0.036	0.038	0.043	0.046
P 1	0.021	0.022	0.028	0.029
P 2	0.001	0.001	0.029	0.030
P 3	0.005	0.005	0.073	0.078
<i>A. NEAPOLITANUS SPP</i>	0.122	0.143	0.032	0.034
<i>A. PICADURUS SPP</i>	0.134	0.157	0.056	0.061

5. CONCLUSIONS

The main object of this research was to analyze diversity patterns of amphipods associated to *Posidonia oceanica* meadows, investigating mechanisms determining their diversity either along spatial and temporal scales or at different level of biodiversity organization (molecular and community).

The studies carried out in this PhD thesis demonstrated that the system *P. oceanica* and associated amphipods is a useful tool to understand patterns and mechanisms that create and maintain biodiversity in the Mediterranean Sea. This is partly due to the relevance of both the “actors” of this study: *P. oceanica* is relevant in Mediterranean Sea as an endemic “foundation species” and Amphipods represent one of the most important groups for diversity and abundance within associated vagile fauna. However, there are other features that have been proved to be relevant in making Amphipods a good case study, namely their low dispersal ability due to the lack of pelagical larvae and their high speciosity.

The relationship between *P. oceanica* and amphipods assemblages are still not well known, particularly regarding the seasonal fluctuations. Therefore the first task of this work has been to understand how amphipods assemblages vary seasonally in terms of difference in species composition and abundance during a one-year survey. Studying temporal variation may help to better understand the relationships with the host plant and if there is a link between *P. oceanica* life cycle and changing in assemblages. In our study, carried on in Giannutri Island, the factor seasonality turned out to be a significant factor in determining changes in the assembly of communities. Although a core of species was present all year long, each season showed its own assemblage characterized by a set of species and these seasonal variations appeared to be linked to the seasonal cycle of the plant. The season with the higher diversity in terms of abundance and richness of species was the warm season (Spring + Summer), when more trophic resources are available. In Autumn, the increase of senescent leaf biomass and the subsequent increase of the debris, both favoured a group of species much more specialized (the most representative being the detritus-feeder *Gammarella fucicola*). In Winter, when *P. oceanica* productivity has its minimum, associated with a drastic decline of trophic resources available, we found the lower diversity in amphipod assemblages. The high number of

species found in *P. oceanica* during the whole year (>60) and the results obtained on assemblage composition, both confirm the hypothesis that seasonal fluctuations in species abundance may reduce niche overlap and suggest the existence of different and complementary time-space based mechanisms behind species coexistence.

To unravel the mechanisms that allow species co-occurrence is an old question in ecology but the possible statistical approaches are now quickly developing, so allowing a deeper investigation of “classical” abundance data. In the second task of this PhD thesis we have therefore used presence/absence data mined from literature to investigate the biogeographical patterns of amphipod communities from meadows over the whole Mediterranean Sea, by applying an innovative method based on network analysis and modularity. This method allowed us to identify the structure of communities and how they grouped according to biogeographic regions understanding which are the main barriers that influence the distribution of amphipods. However, this approach also allowed to deepen the biogeographic analysis by both determining the topological role of localities and identifying the role played by species with different biogeographic origin and range. So the results obtained allowed not only to detect closely related clusters of localities and co-occurring species but also to evaluate the importance of each node in terms of network connectivity, that is not possible with a simple clustering. Our results confirmed a decrease in species richness from West to East, in agreement with the trend found for other Mediterranean vertebrates and invertebrates (Coll. et al., 2010). We also showed the presence of four modules that mainly correspond to four biogeographical regions of the Mediterranean Sea: Central-Western Mediterranean (CWM), Tunisian (TUN), Ionian (ION) and Adriatic (ADR). Each region is delimited by a well-known barrier such as Almeria-Oran Front, Sicily Channel and the Strait of Otranto, which have been pointed out as the most relevant in accounting for by ecological and biogeographical heterogeneity across the Mediterranean Sea. The differentiation among the four modules depends on different aspects of assemblages’ diversity, including richness and range extent. The Central-Western Mediterranean showed the higher diversity, together with Tunisian assemblages and the biogeographical role of localities showed how both these modules could be considered as hubs, i.e. modules that act as sources in a biogeographic context. On the contrary, the Ionian and Adriatic modules resulted peripheral, so characterized by a few, widely distributed species and acting as sinks. Despite amphipods are not exclusively linked to *P. oceanica*, the paleogeographic history of the plants may explain the actual patterns and subdivision of

amphipods assemblages. The area of Tunisia and Sicily Strait has been recently proposed as Glacial refugia, so that the high diversity and the hub role for Tunisian module assemblages could be explained taking into account its role as refugium area.

In the third task of the research, the study was focused on two species belonging to the genus *Apolochus*: *A. neapolitanus* and *A. picadurus*, using a phylogeographic approach. Stated the low vagility of these species and the recent finding of cryptic diversity in many marine amphipods, we have verified the following hypotheses: 1) a pattern of high genetic differentiation associated to cryptic diversity is expected, since both morphological stasis and a genetic architecture favoring high speciation rate have been hypothesized in marine amphipods (Richards et al., 2012; Chenouil et al., 2018); 2) high haplotype and nucleotide diversity should characterize species structure, being the signature of localized refugia when associated to high geographic structuring and to lack of population expansion (Mosblech et al., 2011); 3) deep splits are expected, since the lack of contemporary high gene flow would not have erased the signature of ancient events.

Our result confirmed the presence of high cryptic diversity, showing the presence of at least 6 species in the Mediterranean Sea, 3 in *A. neapolitanus* and 3 in *A. picadurus*, concordantly recovered by all the approaches used. Cryptism in amphipods is not unusual and many authors reported the presence of cryptic species when molecular techniques were implemented (Baird et al., 2011; Beermann et al., 2018; Hupalo et al. 2019). The geographic distribution of these putative species showed that at least some of them were able to cross relevant Mediterranean barriers and to distribute in different seas over the time. However, the remarkable level of genetic structuring here observed both between and within the different biogeographic areas suggests that the levels of ongoing gene flow are low or absent, as witnessed by the lack of shared haplotypes among sampling locations. This observation is only apparently in contrast with the effectiveness of the oceanographic barriers considered, being in substantial agreement with the biological and ecological features of the studied species. The lack of a larval pelagic stage in amphipods, that are brooding species, prevent their massive dispersal through marine currents, so making them fully dependent from rafting or other forms of adult dispersal. As a consequence, both the colonization of new areas and the gene flow occur through rare long distances dispersal events. The signature of such extremely low vagility is a pattern of genetically highly fragmented populations, with comparable differentiation among

populations located both on the same and on the opposite sides of geographic fronts, as here recorded within each *Apolochus* species complex (Pascual et al., 2017).

According to their low vagility and subsequent models of gene flow *Apolochus* species, as other benthic amphipods, have retained the signature of ancient biogeographic events, not overwritten by recent admixture episodes. Correspondingly, the diversification within *Apolochus* morphospecies has been found to be ancient, starting during the Terminal Tethyan Event (TTE, 18-12 Mya, CIT) and following the main Mediterranean paleogeographic vents such as the Messinian Salinity Crisis (5.9-5.3 Mya) and the cyclic ice ages of Plio-Pleistocene. Accordingly with such an ancient history, no populations showed signs of recent expansion.

The results obtained within this PhD thesis provide insights into the usefulness of studying poorly vagile organisms, which are present also in the marine habitats, to assess the biogeographic history of the Mediterranean Sea. Till recent years, the most studied species were characterized by a high vagility, since the main focus was on the phylogeographic break between Atlantic and Mediterranean Sea. More recently the analysis of the biogeographic barriers within the Mediterranean Sea has shown how they are effective in determining both community and genetic structuring within and among species, since the study of organisms with limited dispersal ability allows avoiding the loss of the signature of ancient events because of the superimposition of contemporary high gene flow (Provant and Bennett, 2008). Our results demonstrated that amphipods are among these organisms and suggest that their study may contribute to disentangle the effects of ancient historical events studied by phylogeographic approaches with respect to the role of contemporary barriers, which contribute much more to the co-occurrence patterns within assemblages.

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