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Genetic signature of a recent invasion: The ragged sea hare *Bursatella leachii* in Mar Menor (SE Spain)

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1. Introduction

In the last years, bioinvasions are increasing their ecological and economic impacts on different habitats all over the world, and are therefore becoming the target of much recent research (Ricciardi et al., 2004; Bernardi et al., 2010; Zenetos et al., 2012). The leading hypothesis for the origin of invasions is that colonization by invasive species is most often associated with founder events of introduction by occasional windows of opportunity (Montefalcone et al., 2010; Zhan et al., 2010), although distinct invasion processes might occur simultaneously in different parts of the invaded range, and some can create local accumulation of genetically distinct invaders. In a typical invasion scenario, however, the few founders are then the source of rapid demographic expansions in the new habitats. Such processes result in extreme genetic diversity loss. Biological invasions thus contradict the paradigm of genetic diversity being essential for adaptation to novel habitats.

The Mediterranean Sea has been an important model to study invasive processes, having hundreds of alien species. Most Indo-Pacific species that are currently present in the Mediterranean are considered to have migrated from the Red Sea via the Suez Canal (Lessepsian invaders), since its opening in 1869 (Bernardi et al., 2010). One of these Lessepsian species is the opistobranch *Bursatella leachii* (Blainville, 1817). It is a circumtropical species, widespread along the temperate water of the Indo-Pacific and Atlantic Ocean (Zakhama-Sraieb et al., 2009), and nowadays common in the eastern Mediterranean (Zenetos et al., 2012). The first record from the Mediterranean Sea was found in Palestine (1940) and successively recorded in Turkey

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(1961), Malta (1970), Israel (1970), Sicily (1980), Tunisia (1982), Italy (1984), Slovenia (1989), Greece (1992), Lebanon (collected by G. Bitar and H. Zibrowius, identification confirmed by J. Templado), Sardinia (collected by A. Olita, identification confirmed by J. Templado) and finally in the Southern of Spain (Nicolaidou et al., 2012 and references therein).

As most sea hares, *Bursatella leachii* is a cross fertilizing simultaneous hermaphrodite with internal fertilization (Paige, 1988; Tarikul and Akyol, 2012). The individuals produce large amounts of benthic egg strings and have planktotrophic larvae that complete development in approximately 19 days (Paige, 1988; Tarikul and Akyol, 2012). Their life cycle is short, with maturity at 2–3 months and maximum life expectancy just over one year, and it can only be completed in warm waters, as embryos develop optimally at 20–30 °C, ceasing at 15 °C (Paige, 1988).
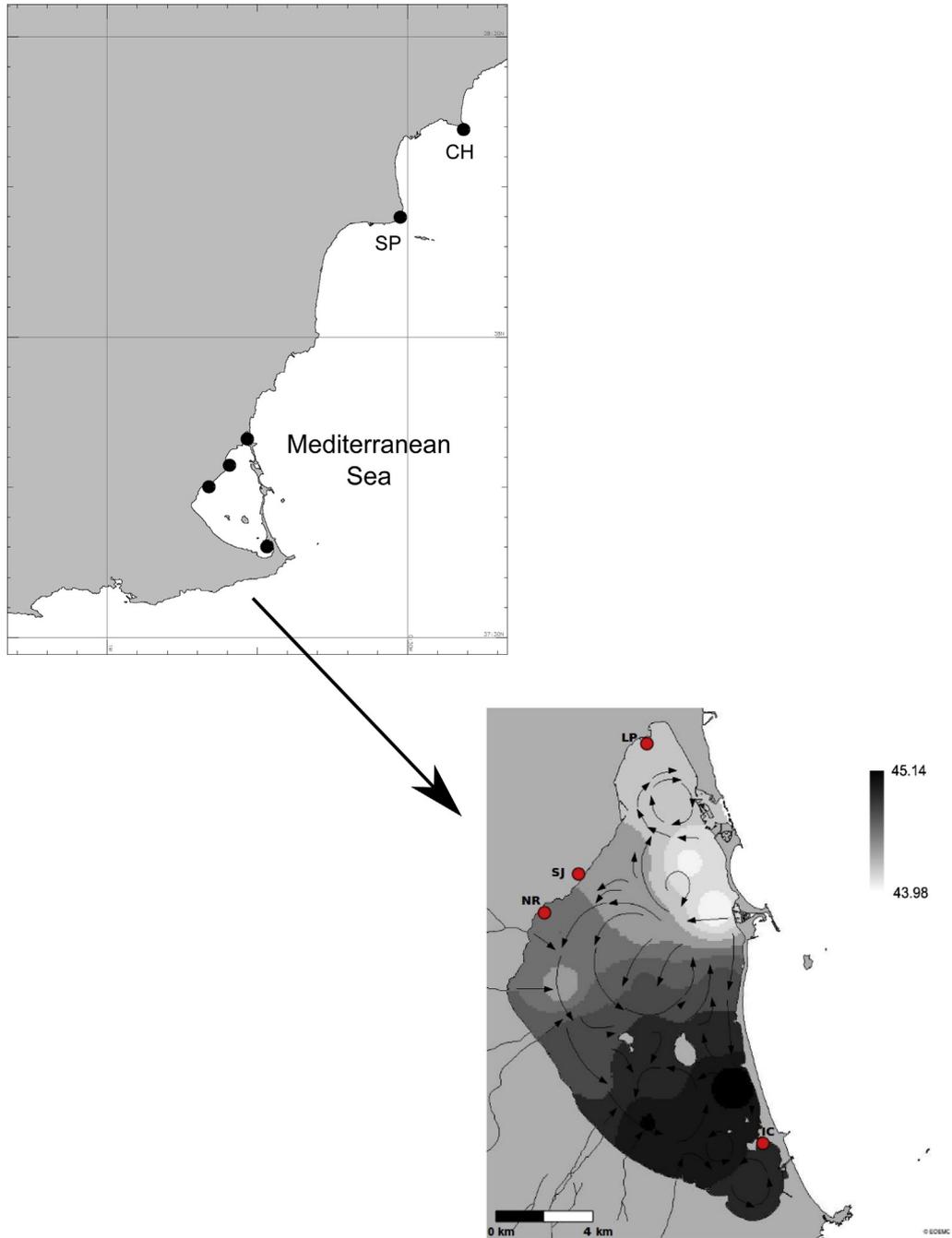

Fig. 1. Sampling sites for *Bursatella leachii*. Mediterranean Sea: CH, Cabo de Huertas; SP: Santa Pola; Mar Menor coastal lagoon: Lo Pagan (LP), San Javier (SJ), Los Narejos (NR) and Isla del Ciervo (IC). The map of Mar Menor shows the interpolated surface of mean salinity and surface currents inside the lagoon (from Gonzalez-Wanguemert et al., 2009).

Like other sea slugs, *B. leachii* is chemically protected from predators by the presence of skin glands which secrete noxious or unpalatable compounds (Rajaganapathi et al., 2002).

B. leachii is highly sporadic in occurrence, spatially and temporally. It is periodically encountered in high densities in the environment, for example densities of 660 individuals per square meter have been reported (Lowe and Turner, 1976; Turk and Furlan, 2011; Tarinkul and Aykol, 2012). These population explosions could be attributable to unpredictable environmental changes which create the optimal conditions for development of this species. Usually, these blooms occur when larval supply is high, food resources are not restrictive, and tides, currents, and weather conditions are favourable.

All these biological features (abundant benthic eggs, planktotrophic larvae with very fast development, maturity at 2–3 months, life expectancy over one year, high densities, blooms, etc) are favouring the fast spreading and high gene flow of *B. leachii* colonizing a new site. Its survival during the colonization event is increased by its chemical defense.

Lessepsian species in general, and *B. leachii* in particular due to forming important blooms, constitute optimal model species to study bottleneck events and subsequent expansions associated to invasions, and to assess possible loss of diversity through the colonization processes. Therefore the aim of this work was to study the genetic diversity and structure of *B. leachii* in Mar Menor coastal lagoon (SE Spain) where in the last three years several population explosions have been occurring, showing densities among 15–100 individuals per m2 (Nicolaidou et al., 2012). The study of this marginal population with high densities at the westernmost edge of the invasion distribution range of *B. leachii*, can be particularly informative in the assessment of its invasive process as was demonstrated for another species (Bernardi et al., 2010). Our results will allow us to assess the existence of founder events (undergone bottlenecks) and to improve the knowledge of colonization/invasion events and dispersal patterns of the Lessepsian species.

2. Materials and methods

2.1. Sampling area and field procedures

The Mar Menor, one of the largest (135 km^2) Mediterranean coastal lagoons, is located on the Southeastern coast of Spain (Fig. 1). The importance of this lagoon and its salt marshes in terms of biodiversity has been recognized by its featuring in numerous international protection schemes (Lloret et al., 2008): it has been a Ramsar International site since 1994; it is considered a Special Protected Area of Mediterranean Interest (SPAMI), established by the Barcelona Convention in 2001; and a Site of Community Importance (SCI) to be integrated in the Nature 2000 Network (EU Habitats Directive). This zone is also a Specially Protected Area (SPA) in relation to nest building, migration and hibernation of aquatic birds, which is protected by European legislation (Birds Directive 79/409/CEE).

The Mar Menor coastal lagoon has a mean depth of about 4 m with a maximum of 6 m. Its waters show a residence time estimated between 0.55 and 1.2 years (Gonzalez-Wanguemert et al., 2009). It is isolated from the Mediterranean Sea by a 22 km long sandy bar (La Manga) which is crossed by 5 channels, being the Estacio the most important channel because it was widened and dredged up to about 4 m deep to make it navigable.

Mar Menor is a hypersaline lagoon, where salinity oscillates between 39 and 47 psu due to high evaporation and low exchange rates with the Mediterranean Sea (Gonzalez-Wanguemert et al., 2009; Vergara-Chen et al., 2013) dependent on the balance of evaporation/rainfall and wind regime. Some temporary watercourses flow to the lagoon, collecting run-off water from the watershed which is mainly used for intensive agriculture (evaporation largely exceeds rainfall and run-off). Water temperature ranges from 10 °C in winter to 31 °C in summer. The bottom of the central area of the lagoon is covered by mud with low hydrodynamism and by dense meadows of the green alga *Caulerpa prolifera*.

Three main basins have been differentiated inside the lagoon on the basis of their hydrographical characteristics (Gonzalez-Wanguemert et al., 2009; Gonzalez-Wanguemert and Pérez-Ruzafa, 2012; Vergara-Chen et al., 2013). The northern basin has the greatest influence of Mediterranean waters through the inlets in the north of La Manga and El Estacio, showing the lowest mean salinity values. The southern basin is a more confined area and it has the most saline water. The central basin has intermediate salinity due to the mixing of Mediterranean and lagoonal waters.

Four sampling sites were selected inside the Mar Menor coastal lagoon distributed along its range and including the three basins previously described (Fig. 1): Isla del Ciervo (IC) on the southern basin, Lo Pagan (LP) and San Javier (SJ) on the northern basin and Los Narejos (NR) located on the central basin. Forty individuals of *B. leachii* were collected from shallow habitats in each locality by snorkeling between October 2010 and January 2012. The samples were transported frozen to the laboratory. Foot muscle tissue was removed from each specimen and preserved in 100% ethanol.

To compare with the genetic diversity inside the lagoon, we also sampled *B. leachii* outside in Santa Pola ($N = 3$) and Cabo de Huertas ($N = 15$) both situated north of Mar Menor coastal lagoon in the Mediterranean Sea (Fig. 1). The Mediterranean samplings were carried out during the winter, therefore a few individuals were found. We also included in the analyses the only sequence available in GENBANK for *B. leachii* (AF156146).

During the sampling, the existence of different colour morphotypes of *B. leachii* inside the coastal lagoon was recorded for all sampled individuals. This information was considered in the genetic analyses to find possible genetic differences among morphotypes.

2.2. DNA extraction, PCR and sequencing

The tissue was dissolved using lysis buffer solution with proteinase K, and total genomic DNA was isolated according to Sambrook et al. (1989). DNA concentration and purity were estimated using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies).

The universal primers HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO1490 (5′-GGTCAACAAATCATAAAGA-TATTGG-3′) (Folmer et al., 1994) were used to amplify a 460 bp fragment of the mitochondrial cytochrome oxidase I (COI) gene. In addition, a fragment of 449 bp, which includes a portion of the 16S rRNA gene was PCR amplified using primers 16Sar-L (5′-CGCCTGTTTATCAAAAACAT-3′) and 16Sbr-H (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al., 1991). For COI, double-stranded DNA was PCR synthesized in 19 μl reactions contained 2 μl amplification buffer (10×), 5 μl of each of the above primers (10 mM), 1.4 μl MgCl^2 (50 mM), 0.16 μl of dNTP mix (25 mM) and 0.5 U Taq polymerase, 1 μl DNA diluted 1:50 or 1:400 in function of the DNA concentration. Amplification proceeded with an initial denaturation at 95 °C for 3 min, then 40 cycles of denaturation at 94 °C for 20 s, annealing to 45 °C for 20 s, and extension at 72 °C for 20 s followed by a final extension at 72 °C for 10 min.

For 16s rRNA, double-stranded DNA was PCR synthesized in 24 μl reactions containing 2.5 μl amplification buffer (10×), 2.5 μl of each of the above primers (10 mM), 1.0 μl MgCl2 (50 mM), 0.2 μl of dNTP mix (25 mM) and 0.5 U Taq polymerase, 1 μl DNA diluted 1:400 of the DNA concentration. Amplification proceeded with an initial denaturation at 95 °C for 3 min, then 40 cycles of denaturation at 94 °C for 20 s, annealing to 45 °C for 20 s, and extension at 72 °C for 20 s followed by a final extension at 72 °C for 10 min.

A 4 μl sample of each PCR product was mixed with 3 μl of gel red, it was run in 2% agarose gel and viewed under Molecular Imager® Gel Doc™ XR+ system (Bio-Rad). Amplified products were sequenced using an ABI Prism 3130 automated genetic analyser (Applied Biosystems) according to the protocols from the Sequencing Service of CCMAR (http://ccmar.ualg.pt/cts).

The sequences were aligned using BioEdit software (Hall, 1999). Intraspecific relationships were established by means of phylogenetic networks using a statistical parsimony method (Templeton et al., 1992) implemented in the software TCS v.1.21 (available at http://darwin.uvigo.es/software/tcs.html). This method determines a 95% statistical confidence limit for the maximum number of nucleotide sites expected to differ between two given haplotypes without any superimposed substitutions, the '95% confidence limit of parsimony' (Templeton et al., 1992). This method displays higher resolution in cases where the level of divergence among sequences is low (Posada and Crandall, 2001), as in the case of intraspecific sequences.

3. Results

3.1. Population genetics

Analysis of 80 individuals from Mar Menor coastal lagoon (20 individuals per locality) with COI mitochondrial gene sequences of 460-bp in length and 16S gene (sequences of 400-bp), detected only one haplotype for each gene (BSCOI-1 and BS16S-1; GENBANK code KJ187403; GENBANK code KJ174529). Individuals sampled outside the lagoon in the Mediterranean Sea showed as most common haplotypes the same present inside the lagoon (BSCOI-1 and BS16S-1), but also two more haplotypes for the COI gene (BSCOI-2 and BSCOI-3; GENBANK codes KJ187404 and KJ187405) with 8 and 10 mutational changes respectively. The COI haplotype available in GENBANK for *B. leachii* (AF156146) showed three mutational changes (Fig. 2).

3.2. Morphotypes

Two colour morphotypes were recorded for *B. leachii* species into Mar Menor coastal lagoon (dark and light, Fig. 3a and b respectively). Of the 160 specimens of *B. leachii* sampled in Mar Menor, 124 individuals were classified as dark and 36 as light morphotypes. Three localities, Isla del Ciervo, Lo Pagán and Los Narejos show mainly individuals with the dark morphotype (92.5%, 95% and 85% respectively), while in San Javier the light morphotype was the most abundant (62.5%). All individuals caught outside the lagoon in the Mediterranean Sea displayed the dark morphotype. Any genetic differentiation was found among morphotypes considering that only one haplotype per each gene was detected in the coastal lagoon.

4. Discussion

The analysis of the sequences from individuals of *B. leachii* from Mar Menor showed only one haplotype for each gene. These results are in strong contrast with the high genetic diversity of other invertebrate species inhabiting this coastal lagoon, such as the 39 haplotypes (COI gene) detected in the bivalve *Cerastoderma glaucum* (Vergara-Chen et al., 2013) or the 32 (COI gene) and 39 haplotypes (16S gene) found in the sea cucumber *Holothuria polii* (Vergara-Chen et al., 2010). *C. glaucum* is a typical lagoonal species, and its high genetic diversity may facilitate its adaptation to the variable and stressful conditions, with high salinity and temperature fluctuations (Vergara-Chen et al., 2013). Although the sea cucumber *H. polii* is a typical marine species, it has also been able to colonize, with high diversity, the extreme environment of Mar Menor (Vergara-Chen et al., 2010), even though echinoderms are considered a group with low capacity to adapt to salinity changes (Lawrence, 1990). Another opistobranch, *Elysia timida*, also showed high levels of genetic diversity (using allozymes as molecular markers) inside Mar Menor with private alleles which were not found outside the lagoon (González-Wangüemert et al., 2006; Giménez-Casalduero et al., 2011).
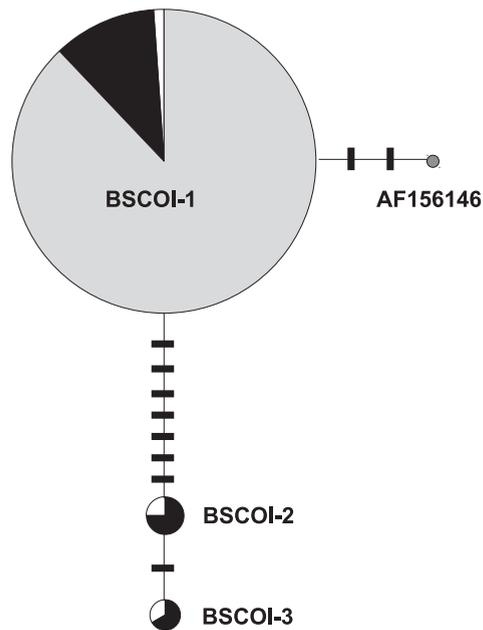
Fig. 2. Statistical parsimony network based on the COI sequences of *Bursatella leachii*. Each haplotype is defined by its corresponding number. The area of each circle is proportional to the number of individuals. Black bars represent putative mutational steps between haplotypes (white area: Santa Pola, SP; black area: Cabo de Huertas, CH; grey area: Mar Menor localities, LP, SJ, NR, IC).

The genetic homogeneity found on *B. leachii* inside Mar Menor could be explained by its invasive dynamics, biological features of the species, the propagule pressure and the environmental conditions. The absence of variation in the mitochondrial DNA loci analysed in this invasive species suggests a genetic bottleneck, hypothetically caused by a founder event due to an introduction by few individuals sharing the same mitochondrial lineage, followed by fast expansion inside Mar Menor. This is favoured by the short life history of the species, with 1 year maximum longevity, its sexual maturity at 2–3 months (Paige, 1988), its presence in high densities which facilitates the internal cross-fertilizing of its simultaneous hermaphrodites and the high temperature of the water in the lagoon during the spring and summer favouring the development of the embryos (González-Wangüemert et al., 2006; Gonzalez-Wanguemert and Pérez-Ruzafa, 2012). The genetic

Fig. 3. Black morphotype of *Bursatella leachii* recorded in Mar Menor and Mediterranean localities (a) and light morphotype found exclusively inside Mar Menor (b).

homogeneity of *B. leachii* inside Mar Menor is also favoured by rapid dispersion, facilitated by its pelagic larvae, the current patterns and the morphology of the lagoon (Gonzalez-Wanguemert et al., 2009). Low diversity associated to bottlenecks by Lessepsian invaders has been reported in some species at the level of the whole Mediterranean Sea (Golani et al., 2007; Bernardi et al., 2010). Although, in the last years is increasing the evidence about that reduced genetic diversity in invasive populations is not as common as expected. Factors as high propagule vectors, and multiple introductions could contribute to the elimination of founder effects in the majority of successful aquatic invasions (Roman and Darling, 2007). The *B. leachii* population is present into Mar Menor 3 years ago, therefore consider multiple introductions and admixture favouring higher genetic diversity during this short time, could be unlikely. However, following studies of this species into the lagoon will can show further changes of the genetic diversity. A possible selection of the more common haplotype guaranteeing better fitness in the lagoon could be also possible, but we did not detect any signature of this event using codon-based Z-test (data not shown).

In spite of the genetic homogeneity of this species in Mar Menor, reducing its adaptive potential, and of its tropical nature, the population of *B. leachii* remains in the lagoon through the years, even during the winter with seawater temperatures below 10 °C (Gonzalez-Wanguemert et al., 2009). This fact could indicate that the introduced haplotypes (one per gene) exhibit plastic responses to novel environments or posses phenotypes with broad environmental tolerances (Sakai et al., 2001; Roman and Darling, 2007). Therefore plasticity and generalism could be considered reasonable hypotheses for the success of low diversity populations (Roman and Darling, 2007).

Also, some behavioural adaptations might be important for the winter survival strategy of the tropical invasive species when are exposed to extreme cold conditions (Turk and Furlan, 2011). In fact, a large group of *B. leachii* individuals was observed (pers. observ.) to aggregate in very shallow waters that were warmer than open seawater during the winter in Isla del Ciervo (Mar Menor lagoon).

A major challenge in understanding invasion processes is explaining the success of an exotic species, not expected to be *a priori* adapted to its novel environmental conditions (Bernardi et al., 2010). However, cases like our *B. leachii* populations, provide an evident example that an extreme bottleneck does not preclude population growth and fast expansion (Golani et al., 2007; Roman and Darling, 2007). Many biotic/abiotic factors and demographic and environmental stochasticity can affect the establishment process, making it difficult to establish the factors of success for invasive species (Lockwood et al., 2007). In the case of Lessepsian invaders, it has been suggested that most had had enough phenotypic plasticity (the ability to cope with a range of environmental conditions) to survive, reproduce and succeed in their novel environment, with 'no need' for evolutionary adaptations (Bernardi et al., 2010). As it was commented before, this could be the case of the *B. leachii*' expansion in the Mar Menor coastal lagoon, where despite the estimated low diversity (only one haplotype for COI and 16s genes) the phenotypes are successful to live and breed under the stressful conditions of the Mar Menor coastal lagoon.

Outside Mar Menor, *B. leachii* contained the most common haplotype from Mar Menor (BSCOI-1) but also two more haplotypes for the COI gene (BSCOI-2 and BSCOI-3), with 8 and 10 mutational changes respectively. Among the haplotypes found outside Mar Menor, the single one found inside can be explained by either a chance colonization event or by differential fitness of this haplotype inside the coastal lagoon. The first hypothesis may be more likely because the haplotype that has colonized the lagoon is the most common one outside. The large number of mutations separating the distinct haplotypes found in the Mediterranean sites, indicates that the separation of those lineages is not recent, and they probably all migrated from the native source population(s) rather than having originated after the invasion or, that multiple introductions on these Mediterranean sites happened favouring a higher genetic diversity. The results also suggest that individuals from the lagoon are breeding inside it, as no other external haplotypes have been found in the lagoonal individuals. In fact, a small population is surviving during the last three years in the south of the lagoon (Isla del Ciervo) which could act as source. This hypothesis about the low gene flow among populations from the Mediterranean Sea and Mar Menor is supported by the low exchange rates of water between seas and the high residence time into the lagoon oscillating between 0.55 and 1.2 years (Vergara-Chen et al., 2013).

We did not find genetic differences between the two *B. leachii* morphotypes. Similar conclusions were reached for *E. timida*, another opistobranch inhabiting the Mar Menor coastal lagoon and the Mediterranean Sea (Gonzalez-Wanguemert et al., 2006). The differences in colouration detected in *E. timida* were linked to its feeding preferences or food availability depending on the algae assemblage for each season or locality (Gonzalez-Wanguemert et al., 2006; Giménez-Casalduero et al., 2011). In the case of *B. leachii*, our observations did not suggest any hypothetical correlation between the morphotypes and the most evident abiotic variables of the habitat; however a differential feeding depending of the available algae, diatoms and bacteria for both morphotypes could be explaining the colour differences. On the other hand, the turbidity of the water and therefore the irradiance reaching on the bottom, could be influencing on the presence/absence of the two morphotypes considering the possible chloroplasts retention on this species such as has been reported for many sacoglossans (Wägele and Johnsen, 2001). Further studies could allow to test these hypothesis.

In conclusion, our findings reaffirm the difficulty of predicting the potential for invasion success and adaptation of a new invader on the basis of its genetic diversity. The *B. leachii*' population inhabiting the Mar Menor coastal lagoon shows genetic homogeneity at mitochondrial genes, but it is able to survive and reproduce with success in the lagoon, having persisted through the years. Future studies using nuclear markers and more samples across the invaded range in the Mediterranean Sea and from the native regions, will be need for a better understanding of its colonization patterns.

Acknowledgements

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