Ascidian introductions through the Suez Canal: The case study of an Indo-Pacific species

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ARTICLE INFO

ABSTRACT

Although marine biological invasions via the Suez Canal have been extensively documented, little is known about the introduction of non-indigenous ascidians (Chordata, Ascidiacea), a group containing particularly aggressive invasive species. Here, we used a multidisciplinary approach to study the introduction of the ascidian *Herdmania momus* into the Mediterranean Sea. We reviewed its taxonomy and global distribution, and analyzed how genetic variation is partitioned between sides of the Suez Canal. The taxonomic revision showed that *H. momus* currently has a wide Indo-Pacific distribution. Genetic data indicated two well-differentiated colonization histories across the eastern Mediterranean. Our findings suggest that the range expansion of *H. momus* has been greatly facilitated by the combined effect of human-mediated transport and the species' ability to adapt to different environments. The integrative approach presented here is critical to attain a holistic understanding of marine biological invasions, especially when studying groups with a poorly resolved taxonomy.

1. Introduction

Over the past two decades enormous progress has been made in the study of biological invasions, greatly improving our ability to understand and predict the spread of non-indigenous species (Richardson and Pyšek, 2008; Geller et al., 2010). A critical step in developing strategies to control and prevent biological invasions is to understand the dispersal pathways by which species are introduced into novel ecosystems. The opening of the Suez Canal initiated an on-going process of marine introductions, mainly from the Red Sea into the Mediterranean Sea (Galil, 2009). The majority of non-indigenous species in the Mediterranean Sea have been introduced this way (Coll et al., 2010; Lejeusne et al., 2010) in a process termed 'Lessepsian migration' (Por, 1978) or 'Erythrean invasion' (Galil, 2000). The magnitude and consequences of such introduction are of increasing concern. For example, more than half of the known Lessepsian migrant fish species have established large populations in the Mediterranean (Goren and Galil, 2005). Some non-indigenous invertebrates have been defined as pests due to the economic damage they have caused fisheries, tourism and power-plant functioning [e.g. the crab *Charybdis longicollis* (Spanier and Galil, 1991) and the jellyfish *Rhopilema nomadica* (Lotan et al.,

1994)]. Although the introduction of several species through the Suez Canal has been well-documented and studied (Galil, 2009), little is known of the nature of these Lessepsian migrations (e.g. life-history traits in the introduced sites, population dynamic, phenotypic plasticity, etc.). In addition to documenting the arrival of introduced species, the application of a variety of analytical and integrative approaches has the potential to significantly advance our understanding of extra-range dispersal of non-indigenous species.

It is generally assumed that the colonization of a new habitat by a species ensues from the introduction of only a small part of the ancestral population, thus creating a 'founder effect' (Mayr, 1942). When a population is affected by a severe reduction in size over several generations it may lose a great part of its original genetic diversity by drift, as demonstrated in studies of insects (Frydenberg et al., 2002), birds (Moum and Arnason, 2001) and fish (Spencer et al., 2000). High levels of genetic diversity in introduced populations have been traditionally considered a prerequisite for successful invasion (Holland, 2000). Nevertheless, numerous studies have also documented bottlenecked populations as highly invasive (Tsutsui et al., 2000; Frankham, 2005; Roman and Darling, 2007). For instance, the blue spotted cornetfish, *Fistularia commersonii*, underwent a severe population bottleneck that considerably reduced its genetic diversity compared to the native populations of the Red Sea (Golani et al., 2007). However, *F. commersonii* has become extraordinarily successful and is rapidly spreading across the Mediterranean Sea. Consequently, careful consideration is

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needed when interpreting genetic data of non-indigenous species (see also Fitzpatrick et al., 2012). In addition, it is imperative to couple genetic information with accurate historical data, which is normally the key for reconstructing the dynamics of biological invasions (Estoup et al., 2010).

In marine systems, ascidians (Chordata, Ascidiacea) contains an increasing number of widely-distributed invasive species (Lambert, 2007; Locke, 2009; Shenkar and Swalla, 2011). Many non-indigenous ascidians are able to tolerate wide fluctuations in temperature, salinity and pollution (Lambert, 2005), have a rapid growth rate, and reach sexual maturity a few weeks after settlement (Lambert, 2001, 2002). A unique characteristic of ascidians is their short-lived, non-feeding planktonic larvae, which represents the only dispersal phase of their life cycle and results in extremely restricted dispersal (Millar, 1971). Therefore, populations found far away from the native range generally represent instances of successful establishment following human-mediated transport. All the above characteristics, combined with high resilience to predation (e.g. Epelbaum et al., 2009), allow some non-indigenous ascidians to become highly successful invasive species. For example, the solitary ascidian, *Microcosmus squamiger*, is abundantly found in harbors and marinas worldwide (Rius et al., 2012), but it can also spread outside these habitats, altering local benthic communities by forming dense and extensive aggregates (Rius et al., 2009a). Ascidians have repeatedly shown their ability to outcompete sessile organisms and alter ecosystem functioning in numerous ways in both temperate and tropical environments (e.g. Castilla et al., 2004; Bullard et al., 2007; Shenkar et al., 2008a; Rius et al., 2009b). The introduction of these organisms affects human activities (Locke and Carman, 2009), with aquaculture being one of the most heavily impacted activities (Ramsay et al., 2008; Rius et al., 2011).

There are several introduced ascidian species in the Mediterranean Sea that are suspected of being Lessepsian migrants (Por, 1978; Izquierdo-Muñoz et al., 2009; Shenkar and Loya, 2009), but little information is available on how artificial canals modulate the introduction of such species into new territories. Here, we focused on the solitary ascidian *Herdmania momus* (Savigny, 1816), an Indo-Pacific species that has most likely been introduced into the Mediterranean through the Suez Canal (Shenkar and Loya, 2008, 2009). *H. momus* was first recorded in the Suez Canal in 1924 (Harant, 1927), while the first record in the eastern Mediterranean was in 1958 (Pérès, 1958). Since then, *H. momus* has been detected in Cyprus (Monniot, 2002; Nishikawa, 2002), Turkey (Çinar et al., 2006) and Lebanon (Bitar et al., 2007). In contrast, this species has never been reported in the western basin of the Mediterranean Sea or the Adriatic Sea (Izquierdo-Muñoz et al., 2009). Currently, this species is a conspicuous occupant of artificial structures along the Mediterranean coast of Israel (Shenkar and Loya, 2008, 2009), and is rarely found in natural environments (Shenkar et al., 2008b). Our objectives were to: (1) Review and clarify the taxonomy of *H. momus*; (2) Obtain historical information from taxonomic records to demarcate the species range of *H. momus*; (3) Analyze how genetic variation is partitioned between the two sides of the Canal. We first reviewed its taxonomy, colonization history, and global distribution, and subsequently explored and compared genetic diversity and population structure on both sides of the Suez Canal.

2. Materials and methods

2.1. Taxonomic data

We reviewed all reports of *H. momus* in the literature. We also considered reports of close relative species within the genus *Herdmania* that might have been misidentified as *H. momus*. Our aim

was to indicate the taxonomic features that discern *H. momus* from other species within its genus.

2.2. Global distribution data

In order to ascertain the global distribution of *H. momus* we conducted a thorough review of the taxonomic records. Whenever a taxonomic description was incomplete or corresponded to more than one species, and no confirmation could be obtained from a specialist, we categorized the geographic position of the taxonomic record as unconfirmed. We reviewed all studies that have reported this species since the opening of the Suez Canal in 1869.

2.3. Genetic analysis

We surveyed sites on both sides of the Suez Canal where *H. momus* was present and permission to collect samples could be obtained (Fig. 1, Table 1). These sites included two from the Red Sea [Eilat's oil port, which is a natural protected coral reef; and a former location of fish cages in Eilat, near the Jordanian border (see Loya, 2007)], and four from the Mediterranean Sea (Hadera electric company pier, artificial reefs in Shikmona and Achziv, and Famagusta harbor). There are marked differences in environmental conditions between the tropical Red Sea and the temperate rocky shores of the Mediterranean Sea. The water temperature in Eilat varies from 20 to 27 °C and salinity is ca. 40.5‰ (Loya, 2004). Along the Mediterranean coast of Israel the water temperature varies from 17 to 30 °C and salinity is ca. 39.0‰ (Por, 1978). Samplings were performed by SCUBA or snorkeling throughout
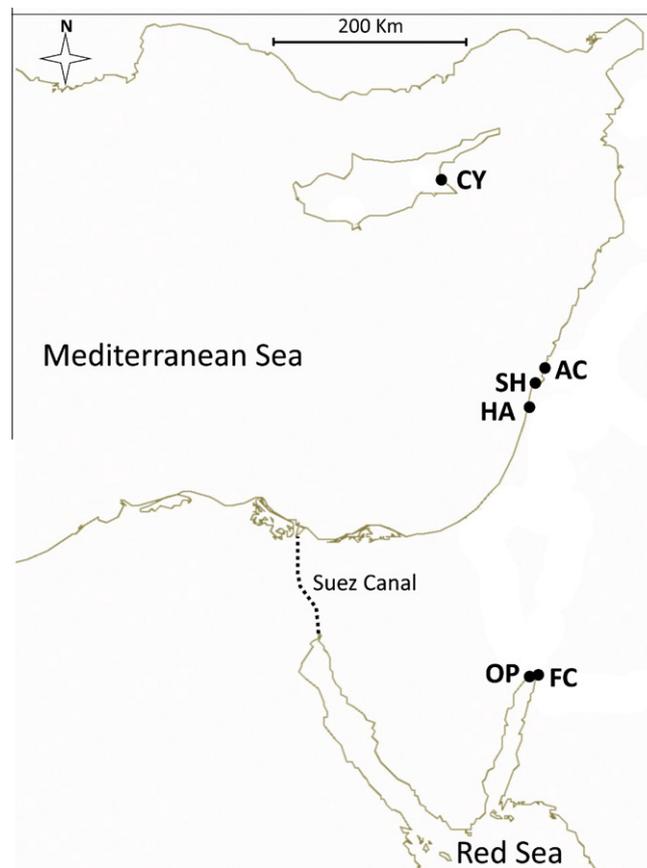

Fig. 1. Map of the studied region with the position of the sampled sites and the Suez Canal indicated. Site name abbreviations as in Table 1.

Table 1
Characteristics of the *H. momus* sampled sites.

Site	Abbreviation	Latitude/longitude	*n*	Nh	Uh	*h* ± S.D.	*π* ± S.D.	Np
Eilat, fish cages	FC	29°30′N/34°55′E	10	6	2	0.778 ± 0.137	0.004 ± 0.001	8
Eilat, oil port	OP	29°31′N/34°56′E	20	5	1	0.663 ± 0.095	0.004 ± 0.004	8
Hadera	HA	32°28′N/34°53′E	20	8	2	0.837 ± 0.059	0.010 ± 0.002	21
Shikmona	SH	32°49′N/34°50′E	12	6	2	0.848 ± 0.074	0.011 ± 0.002	17
Achziv	AC	33°03′N/35°06′E	14	6	1	0.791 ± 0.089	0.015 ± 0.002	26
Famagusta harbor, Cyprus	CY	35°08′N/33°56′E	20	2	0	0.100 ± 0.088	0.001 ± 0.000	3
Total			96	16		0.782 ± 0.029	0.009 ± 0.001	

Sampled size (*n*), number of haplotypes (Nh), number of private haplotypes (Uh), haplotype diversity (*h*) and nucleotide diversity (*π*) with their standard deviation (S.D.), and number of polymorphic sites (Np).

2006. All samples were kept in absolute ethanol at −4 °C until used.

DNA was extracted from gonads that were removed from the mantle by dissection. DNA extraction was performed using Biosprint 96 (Qiagen) and DNA was eluted in 200 μL Qiagen elution buffer and stored at −20 °C. We used a fragment of the mitochondrial cytochrome c-oxidase subunit I (COI) gene, which has proven to be highly informative for both intra and interspecies studies in ascidians (e.g. Turon and López-Legentil, 2004; López-Legentil et al., 2006; Teske et al., 2011). COI amplification was carried out on all samples in a 96-well polymerase chain reaction (PCR) plate using Meyer (2003) degenerated primers (dgLCO1490) 5′–GGTCAA CAAATCATAAAGAYATYGG–3′ and (dgHCO2198) 5′–TAAACTTCAGG GTGACCAAARAAYCA-3′ (*Y* = *C* or *T*, *R* = *A* or *G*). For the amplification reaction a total volume of 10 μL was used, comprising 1 μL 10× buffer (Bioline, without MgCl₂), 0.3 μL of each primer (10 mM), 0.5 μL MgCl₂ (50 mM), 0.25 μL of BSA (100× New England Biolab), 0.5 μL dNTPs (10 mM, Bioline), 0.1 μL Taq polymerase (Bioline), 6.05 μL dH₂O and 1 μL DNA template. Cycling conditions consisted of an initial denaturing step of five minutes at 95 °C followed by 34 cycles (30-s denaturing at 95 °C, 30-s annealing at 48 °C, and 45-s elongation at 72 °C), and a final elongation step of 7 min at 72 °C. PCR products were cleaned using ExoSAP-IT®Affymetrix Inc., with 2 μL of fourfold diluted ExoSAP-IT. Sequencing reaction using both primers was performed with 0.5 μL big dye (Applied Biosystems) and 1 μL template per reaction and analyzed on an ABI 3730 96-capillary machine.

The sequences were aligned using the BioEdit Sequence Alignment Editor v.7.0.5.2 (Hall, 1999) and the resulting alignment was verified manually. Nucleotide sequences were translated into amino-acid sequences using the ascidian mitochondrial genetic code. The DnaSP v.5.10 software (Librado and Rozas, 2009) was used to obtain the number of haplotypes, the standard diversity indices (haplotype and nucleotide diversities) per sampled site (Nei, 1987), the number of unique haplotypes, and the number of polymorphic sites (synonymous and non-synonymous substitutions). A haplotype network was generated using the TCS program v.1.21 (Clement et al., 2000), which creates an absolute distance matrix by calculating all possible pairwise comparisons among haplotypes, considering a parsimony probability of 0.95 (Templeton et al., 1992). To create a phylogenetic tree of the haplotypes found we used the program MEGA v.4.1 (Kumar et al., 2008). We used the UPGMA method with the Maximum Composite Likelihood as the best-fit model of nucleotide evolution for our data, and calculated the branch support with bootstrap test (10,000 replicates). A sequence of the congeneric species *Herdmania grandis* (GenBank ID: FJ528630.1) was used to root the tree.

To analyze genetic structure, we used Φ_{ST} that includes the extent of sequence divergence among haplotypes. We implemented the exact test of population differentiation (10,000 permutations) to test the hypothesis of random distribution of the individuals between pairs of sampled sites using Arlequin v.3.5.1 (Excoffier and Lischer, 2010). We used false discovery rate (Benjamini and Yekutieli, 2001) to correct for multiple tests. Recent studies have shown that G_{ST} and its variants do not accurately reflect genetic differentiation among populations when variation within populations increases (Jost, 2009). Thus, in addition to Φ_{ST}, we calculated the genetic diversity measure *D* defined by Jost (2008), which measures the relative degree of differentiation of allele frequencies across populations. We calculated pairwise *D* values, the confidence intervals and the global *D* using the program SPADE (Chao and Shen, 2009). A non-metric multi-dimensional scaling (MDS) ordination was carried out with the program Primer [v.6.1.11, PRIMER-E Ltd., (Clarke and Gorley, 2006)] using the matrix of *D* as an input to graphically represent the patterns of genetic distances. In addition, we ran a neighbor-joining tree computed by the program MEGA using the matrix of *D* as input. In order to test the presence of isolation by distance among sampled sites, we evaluated the relationship between the matrices of genetic differentiation (either using Φ_{ST} and *D*) and geographical distances (as the shortest continuous distance by sea in km) between sampled sites using a Mantel test (Mantel, 1967) as implemented in Arlequin with 10,000 permutations. Subsequently, we conducted a hierarchical analysis of molecular variance (AMOVA) using Arlequin to study the distribution of genetic variability among localities (significance of the variance components tested by 10,000 permutations). The sampled sites were grouped with the following *a priori* expectations according to their status: (1) Native vs. introduced, and (2) Native vs. Mediterranean coast of Israel vs. Cyprus.

3. Results

3.1. Taxonomic data

H. momus was formally described by Savigny (1816) based on specimens from the Red Sea. Since then, several species have been misidentified as *H. momus*, as indicated in recent reviews (Kott, 2002; Monniot, 2002; Nishikawa, 2002). For example, the closely-related species *Herdmania pallida* has often been confused with *H. momus*, most likely due to their similar external appearance characterized by a pinkish tunic (Fig. 2).

The species within the genus *Herdmania* differ with regard to how the mantle musculature is distributed, and the shape of the gut, the hepatic gland, the gonads and the gonoducts (Monniot, 2002). *H. momus* is distinguishable from all other species within the genus *Herdmania* by its contorted ovary, multiple openings of small sperm ducts, and a lobed anus (Monniot and Monniot, 2001; Monniot, 2002). In contrast, *H. pallida* has no membranous accessories around the orifice of the oviduct. In addition, according to Michaelsen (1919), who considered the American *Herdmania* as representatives of the 'forma pallida', *H. pallida* has a smooth anus margin (while *H. momus* is clearly lobed), and only eight or nine branchial folds in comparison to the nine plus one small fold in
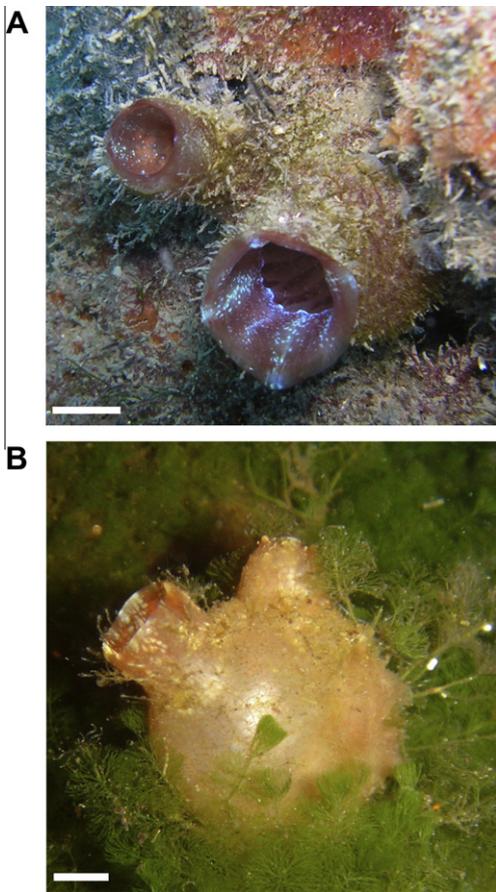
Fig. 2. (A) *H. momus* from the Mediterranean coast of Israel, and (B) the closely-related species *H. pallida* from Bocas del Toro, Panama. Photographs: N. Shenkar. Scale bar 1 cm.

H. momus. However, Nishikawa (2002) showed that the number of branchial folds varies between seven and 10 in *H. momus*. It has recently been suggested that the *Herdmania* spp. found in the Western Atlantic belongs to *H. pallida* and not *H. momus* (Rocha et al., 2012). We found that taxonomists generally agree that the most informative morphological description by which to accurately distinguish *H. momus* from other *Herdmania* species is that given by Nishikawa (2002) and Monniot (2002).

3.2. Global species range

H. momus has a wide tropical Indo-Pacific distribution (Table 2). The northernmost record of this species is along the coast of Turkey (Çinar et al., 2006). Although commonly found along this coast, it has not been observed in the Aegean Sea (M.B. Yokes pers. comm.). Despite the numerous records of *H. momus* from the Western Atlantic coast (Van Name, 1945; Goodbody, 1993), ascidian taxonomists believe that these records refer to *H. pallida* (Monniot, 2002; Rocha et al., 2012). The sole record of *H. momus* from the eastern Atlantic ocean is from Millar (1956), but could not be confirmed because the description was ambiguous and could correspond to more than one species. The global distribution and associated taxonomic literature of *H. momus* are presented in Table 2.

3.3. Genetic characterization

We obtained sequences of a final alignment of 603 bp. The alignment was unambiguous and contained no insertions or

Table 2
H. momus global distribution, references, and comments on the current status of taxonomic records.

Region and taxonomic record	Reference	Comments
Pacific Ocean		
Hawaii	(Abbott et al., 1997)	
Korea Strait	(Rho, 1971)	Record unconfirmed
Japan	(Nishikawa, 2002)	
Australia	(Kott, 2002)	
Fiji	(Kott, 1982)	
French Polynesia	(Herdman, 1882, 1886)	
New Caledonia	(Monniot, 1992)	
Indonesia	(Herdman, 1886)	
Singapore	(Millar, 1975)	Record unconfirmed (see Kott, 2002)
Indonesia	(Millar, 1975)	Record unconfirmed (see Kott, 2002)
Hong Kong	(Kott and Goodbody, 1982)	
Tasmania	(Kott, 1952, 1985; Primo and Vázquez, 2008)	Record unconfirmed
Indian Ocean		
Red Sea	(Savigny, 1816; Shenkar and Loya, 2008)	
Kenya	(Millar, 1988)	
Somalia	(Millar, 1988)	
Mozambique	(Millar, 1956; Nishikawa, 2002)	
Yemen	(Monniot, 2002)	
Bahrain	(Monniot and Monniot, 1997)	
Gulf of Aden	(Nishikawa, 2002)	
South Africa	(Herdman, 1882; Primo and Vázquez, 2004)	Record unconfirmed
Atlantic Ocean		
Caribbean coast of Panama, St. Croix, St. Thomas	(Van Name, 1945)	*H. pallida*[*]
Cuba	(Van Name, 1945)	*H. pallida*[*]
Jamaica	(Van Name, 1945; Goodbody, 1993)	*H. pallida*[*]
Brazilian coast (near Rio de Janeiro)	(Van Name, 1945)	*H. pallida*[*]
Tahiti	(Nishikawa, 2002)	
Mediterranean Sea		
Israel	(Shenkar and Loya, 2009)	
Suez Canal	(Hartmeyer, 1915)	
Lebanon	(Nishikawa, 2002)	
Cyprus	(Nishikawa, 2002)	
Turkey	(Çinar et al., 2006)	

[*] See Monniot (2002) and Rocha et al. (2012).

deletions. The dataset comprised 16 haplotypes (Table 1, GenBank ID: HM490289-HM490304), being eight haplotypes unique (Table S1). Two haplotypes were present in both the introduced and native ranges, two were only shared between the native sampled sites, while the remaining haplotypes were only found in the introduced range (Fig. 3A, Table S1). Most of the sampled sites showed a similar number of haplotypes (five to eight), except Cyprus, which revealed only two non-unique haplotypes. As a result, haplotype and nucleotide diversities were the lowest for Cyprus, while the remaining sampled sites showed similar values (Table 1).

We found 33 (5.4%) polymorphic (segregating) sites, mostly synonymous substitutions, and only one with a non-synonymous substitution (Fig. 3A). We recorded most of these polymorphic sites across the Mediterranean Sea, while the Red Sea showed very low levels of polymorphism (Table 1). Regarding the number of haplotypes, number of polymorphic sites, and number of unique haplotypes, no significant differences were found between the
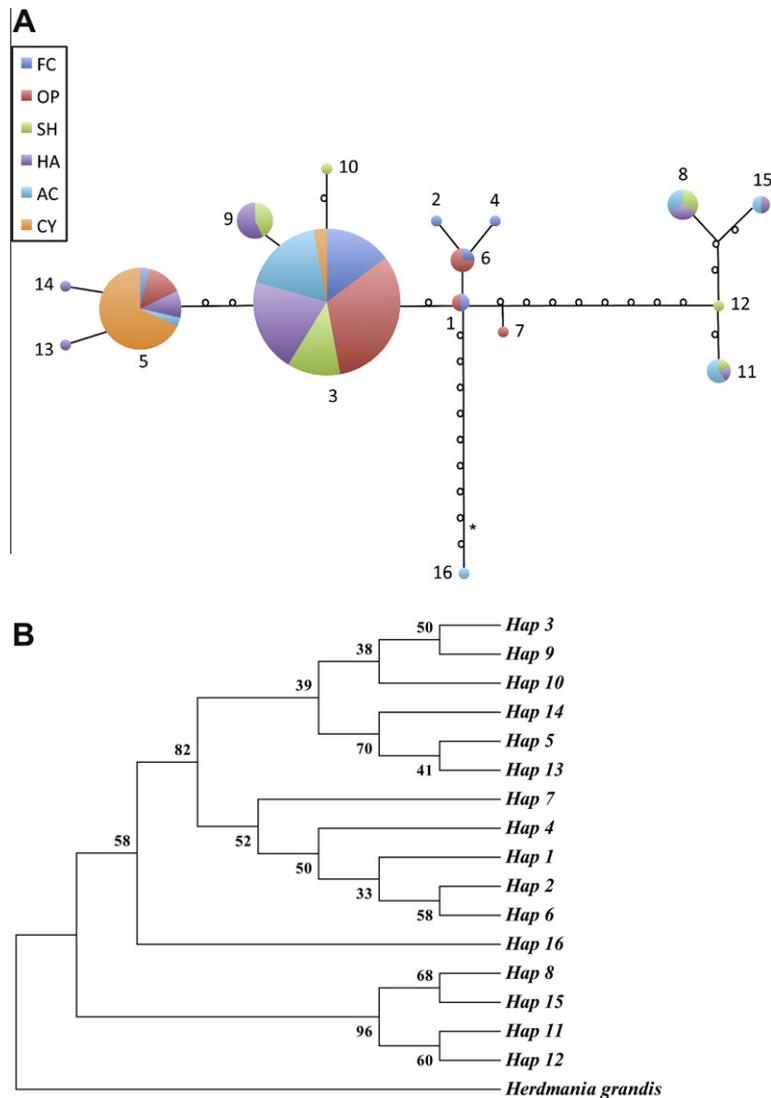
Fig. 3. (A) Haplotype network for *H. momus* representing each sampled haplotype (i.e. each pie chart) with the haplotype number indicated. The size of the pie charts is proportional to the frequency of each haplotype across all sampled individuals. Lines between circles show one mutational step, the smallest circles represent unsampled or extinct haplotypes and * non-synonymous substitutions. Site name abbreviations as in Table 1. (B) Phylogenetic tree of the haplotypes found including an outgroup using the UPGMA method. Numbers indicate bootstrap values (calculated as percentage). Haplotype (*Hap*) numbers are the same as in (A).

Table 3
Genetic differentiation indices for pairwise comparisons of sampled sites of *H. momus*. The Φ_{ST} and D values are shown below and above the diagonal respectively. Site abbreviations as in Table 1. Significant differences between site pairs ($P < 0.05$) are indicated in **bold**.

Φ_S/D	FC	OP	HA	SH	AC	CY
FC	–	−0.127	0.014	0.108	−0.027	**0.786**
OP	−0.023	–	0.110	0.249	0.084	**0.648**
HA	0.082	0.075	–	−0.192	−0.018	**0.699**
SH	0.160	**0.214**	−0.005	–	−0.024	**0.968**
AC	0.188	**0.252**	0.048	−0.036	–	**0.839**
CY	**0.712**	**0.580**	**0.361**	**0.589**	**0.507**	–

native and the introduced sites (*t*-test, $P > 0.175$ in all cases). When we analyzed the distribution of haplotypes, two haplotypes were the most frequent (haplotype numbers 3 and 5, see Fig. 3A) and, interestingly, they were the only haplotypes found in Cyprus. The haplotype phylogenetic tree (Fig. 3B) showed a consistent outcome with the haplotype network.

When we analyzed the genetic differentiation between sampled site pairs, we found the highest Φ_{ST} values in pairwise comparisons

between Cyprus and each of the remaining sites (average of all Φ_{ST} values that included Cyprus = 0.550) (Table 3). The values between the native sites and among the Mediterranean sites (excluding Cyprus) were the lowest (mean of −0.023 and 0.109 respectively). When we tested for differentiation between sampled site pairs we found significant differences ($P < 0.05$) between Cyprus and all other sites, but also between the Eilat oil port and two Mediterranean sites (see Table 3). We found non-significant differences among the native sites as well as among the Mediterranean sites, excluding Cyprus. When we obtained D for each sampled site pair comparison (Table 3) we consistently found the highest values between Cyprus and the other sites. Furthermore, the confidence intervals of pairwise comparisons indicated that the hypothesis of no differentiation could only be rejected for the comparisons between Cyprus and the other five sites. The global value of D was 0.452.

The MDS plot based on D values (Fig. 4A) showed a distribution of the sampled sites in which Cyprus was clearly separated from both the native and other introduced sampled sites, which appeared closely together. The native sites were clearly differentiated in the neighbor-joining tree, while the Mediterranean sites were
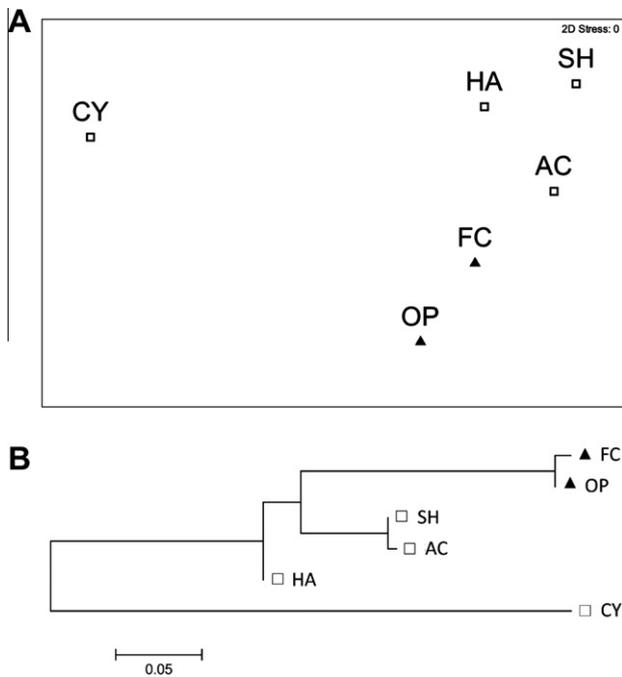
Fig. 4. Genetic distances (using *D* values) among the sampled sites of *H. momus* using: (A) Multidimensional scaling ordination, and (B) neighbor-joining tree. Symbols on the plot indicate: native (▲), and introduced (□) ranges. Site name abbreviations as in Table 1.

split into two clusters (Fig. 4B). The Mantel test showed non-significant results ($R = 0.339$, $P = 0.101$ using Φ_{ST}; $R = 0.076$, $P = 0.768$ using D), indicating the absence of a significant correlation between geographic and genetic distances.

The AMOVA analysis showed significant differences in genetic diversity among groups when we included three groups: the native sites, Cyprus, and the remaining Mediterranean sites (Table 4). In contrast, we found non-significant results when the native sites were compared to all non-indigenous sites grouped together. This was mainly due to the high variability within groups, which resulted in significant differences among sampled sites within groups. Finally, we found that for the two analyses a considerable amount of variance was explained by the variation within sites (Table 4).

4. Discussion

Our study combined taxonomic, biogeographic, and genetic data to understand the arrival of *H. momus* into the Mediterranean Sea. This multidisciplinary approach provided two primary sources of information: (1) The taxonomic review resulted in a comprehensive overview of the global range of the studied species, which currently comprises predominantly of regions along the coasts of the Indian and Pacific oceans, as well as the Mediterranean Sea; (2) The patterns of genetic diversity and population structure around the Suez Canal revealed dissimilar colonization histories of the different regions studied along the eastern Mediterranean coast. The approach presented here is especially critical for groups whose taxonomic status is unresolved, as well as to discern between native and introduced ranges.

Both physical and biotic conditions in the introduced range are usually different from those in the native environment, generating a challenge for possible colonizers seeking to adapt to the new environment. In order to meet both the ecological and the trophic requirements for survival in the Mediterranean Sea, introduced populations of *H. momus* must possess a broad adaptive plasticity. Such plasticity has been demonstrated in the significant meristic changes and spawning season modification of some introduced fishes (Golani, 1990). Phenotypic plasticity has most likely played an important role in the successful adaptation of *H. momus* to its new environment, as demonstrated by its larger body size in the Mediterranean and ability to amend its reproduction (Shenkar and Loya, 2008). The reproductive phenology of *H. momus* in the Mediterranean Sea shows that gamete release only occurs when water temperature reaches similar values to those of the Red Sea (around June and November). This strong seasonal pattern is similar to that of other ascidians found in this sea (Becerro and Turon, 1992; Rius et al., 2009a). In contrast, and as seen in other tropical ascidians (Goodbody, 1961; Van Duyl et al., 1981), in the Red Sea *H. momus* reproduces year round (Shenkar and Loya, 2008). This implies that multiple overlapping generations can be found in the same year. Its spread across tropical seas is therefore more likely to be successful due to its year-round reproductive activity.

Our study contributes to a growing body of literature demonstrating that non-indigenous species are expanding their distributional ranges (Locke, 2009; de Rivera et al., 2011). Historical records, together with the findings on global distribution, suggest that populations of *H. momus* are expanding both globally and across the eastern Mediterranean. We found that the colonization of Mediterranean regions has followed a geographic sequence as evidenced by the fact that most of the recent records (Nishikawa, 2002; Çinar et al., 2006) are from the regions located furthest from the Suez Canal. While transoceanic shipping is responsible for such spread, the consequences of the associated translocation of species remain to be fully understood. Human-mediated transport is responsible for homogenizing the genetic composition of distant non-native populations (e.g. Pineda et al., 2011; Rius et al., 2012).

Table 4
Analysis of molecular variance for *H. momus* considering different groupings of the sampled sites, and based on prior expectations considering their status and geographic location.

Grouping	Source of variation	SS	d.f.	% V	Variance components	Fixation indices
1. Native sites	Among groups	14.771	1	1.19	0.033 Va	F_{CT}: 0.012
2. Non-indigenous sites	Among sites within groups	50.026	4	24.14	0.672 Vb	F_{SC}: 0.244[*]
	Within sites	187.119	90	74.67	2.079 Vc	F_{ST}: 0.253[*]
	Total	251.917	95		2.784	
1. Native sites	Among groups	55.909	2	27.71	0.820 Va	F_{CT}: 0.277[*]
2. Cyprus	Among sites within groups	8.889	3	2.07	0.061 Vb	F_{SC}: 0.029
3. Sites along the Mediterranean coast of Israel	Within sites	187.119	90	70.22	2.079 Vc	F_{ST}: 0.298[*]
	Total	251.917	95		2.961	

Sum of squares (SS), degrees of freedom (d.f.), percentage of variation (% V) are indicated for each grouping. Va, Vb, and Vc are the associated covariance components. F_{SC}, F_{ST}, F_{CT} are the *F*-statistics.
[*] Significant differences ($P < 0.05$).

Yet, little is known about the effects of such transport on selection regimes of these organisms (e.g. Mooney and McGraw, 2007). The inherited characteristics of many ascidians ensure that they easily adapt to multi-climatic conditions, as shown here with *H. momus*. However, further studies are needed to unravel the long-term consequences of global species transfers. To date, the data accumulated show that *H. momus* has sustained viable populations in the Mediterranean. These populations have not yet been shown to outcompete native species or invade natural ecosystems (Shenkar et al., 2008b). Therefore, we consider that at present this species sustains naturalized but not invasive populations (sensu Richardson et al., 2000).

We found evidence of two well-differentiated processes driving the introduction of *H. momus* into the Mediterranean. The first involves the sampled sites of the Mediterranean coast of Israel where high levels of haplotype and nucleotide diversity were found. The second process involves *H. momus*' introduction into the Famagusta harbor in Cyprus, which was characterized by low genetic diversity and the absence of unique alleles. Concerning the first event, genetic studies have revealed little or no genetic differentiation between the source and introduced populations of several Lessepsian species, showing a fairly continuous gene flow (Bucciarelli et al., 2002; Hassan et al., 2003; Hassan and Bonhomme, 2005). Accordingly, a low degree of genetic differentiation was found in *H. momus* between (and within) the native (Red Sea) and non-indigenous sites along the Mediterranean coast of Israel. In addition, no significant differences were found in number of haplotypes, number of unique haplotypes, or in number of polymorphic sites between native and introduced sites. Although we found high levels of genetic variability within sampled sites in the AMOVA results, they showed that the sampled sites of the Mediterranean coast of Israel had little genetic differentiation and shared rare haplotypes, which suggests that colonization along this coast is likely to have occurred non-independently. Human-mediated transport can enhance both the number of individuals reaching new areas, and the probability of multiple introductions (Roman and Darling, 2007), which consequently increases genetic variability and reduces bottleneck effects. The relatively high number of unique haplotypes found along this coast, together with the high levels of genetic diversity, suggest that *H. momus* was introduced into this region either as the result of a single introduction of a large number of genetically heterogeneous individuals, or of recurrent introductions from the Red Sea. At the Hadera electric company pier, approximately 25% of the cargo ships arriving every year have previously passed through the Suez Canal, while Haifa port (adjacent to the Shikmona study site) receives significantly higher cargo traffic every year (2500–2700 ships annually), of which a considerable proportion have passed through the Suez Canal (R. Tzadok, EcoOcean). Consequently, it is highly likely that *H. momus* has been introduced to the Mediterranean coast of Israel on numerous occasions and/or in large numbers. Similarly, studies on introduced fishes (Bonhomme et al., 2003; Hassan et al., 2003; Hassan and Bonhomme, 2005) and mussels (Shefer et al., 2004) suggest that high levels of propagule pressure and gene flow have enabled their establishment in the Mediterranean Sea.

The introduction of *H. momus* to Cyprus is characterized by the lowest levels of haplotype diversity across sampled sites, with the haplotypes found there being the two most common ones in the haplotype network (Fig. 3A). This suggests that in Cyprus *H. momus* either underwent a severe population bottleneck, as seen in other ascidian species (Pérez-Portela et al., 2012), or that the genetic composition of the sampled site originated from a single founder event of genetically non-diverse individuals. The first record of *H. momus* in Cyprus was from Famagusta harbor in 1998 (Monniot, 2002; Nishikawa, 2002). By September 2005, *H. momus* was documented as forming large aggregations on the walls of this harbor

(I. Mizrachi, unpublished data). In agreement with the geographic and temporal sequence suggested by the taxonomic records, genetic diversity decreased with distance from the canal. Based on both genetic and taxonomic data, we conclude that Cyprus is most likely a recent introduction compared to the other introduced sites.

In contrast to colonial ascidian species, which are usually ovoviviparous, in solitary ascidians such as *H. momus* fertilization and larval development occur in the water column (Berrill, 1950). Therefore, it is possible that *H. momus* could have naturally dispersed through the Suez Canal via water currents during its gamete and/or larval dispersal phase. However, the marked absence of *H. momus* from natural substrates along the Mediterranean coast of Israel (Shenkar and Loya, 2008), as well as from harbors and artificial structures found among the sampled sites (e.g., Ashdod electric company pier, N. Shenkar, unpublished data), suggests that the hypothesis of a natural larval drift to the eastern Mediterranean basin is less likely. Since the lecithotrophic larval stage of solitary ascidians is short, the primary mode of ascidian introduction is through the transportation of adult forms growing on boat hulls or other fouled surfaces such as sea chests (e.g. Carlton and Geller, 1993; Lambert, 2002; Coutts and Dodgshun, 2007). Adult ascidians on ship or barge hulls may survive transport over thousands of kilometers to harbors possessing conditions similar to those found within the native range (Lambert, 2002). Once the ship stops in a harbor, the ascidians can spawn in a sheltered environment where fertilization of gametes occurs in the water column (Bullard and Carman, 2009). The non-significant results of the Mantel test led us to reject any association between genetic differences and geographic distance. These results, combined with our genetic evidence of non-independent introductions along the Mediterranean coast of Israel, indicate that shipping has been the major dispersal vector of this species.

Genetic studies provide a valuable tool for the study of biological invasions, mainly by identifying source populations and tracking possible introduction pathways (Holland, 2000; Estoup and Guillemaud, 2010). However, caution must be taken when interpreting genetic data of biological invasions. Most data on non-indigenous species violate the underlying assumptions of population structure estimates and migration rates (Fitzpatrick et al., 2012). This is mainly due to the fact that, for most introductions, insufficient time has spanned since their introduction (a few to a few hundred generations), so that mutation rates and drift have yet to reach equilibrium. This has implications for our current findings on population structure, which should be carefully interpreted. The marked seasonality of the reproductive cycle of *H. momus* suggests that at least one non-overlapping generation is produced each year in the Mediterranean. If we consider the opening year of the Suez Canal as the first date when *H. momus* could have become a Lessepsian migrant, a minimum of 137 generations would have occurred between its initial establishment and the time of sampling for the present study. This number of generations might be insufficient to satisfy the above underlying assumptions. In contrast, our findings from the comparison of genetic estimators comparing introduced and native ranges, as well those distinguishing between single and multiple invasion scenarios, remain robust.

Despite the accumulating knowledge worldwide of the harmful effects of non-indigenous ascidians on natural fauna (Lambert and Lambert, 1998; Cohen et al., 2005; Bullard and Carman, 2009; Shenkar and Swalla, 2011), and the growing number of nuisance species of Indo-Pacific origin in the Mediterranean (Coll et al., 2010; Lejeusne et al., 2010), this is the first study to focus on the arrival and spread of a Lessepsian ascidian species. Considering the increase of shipping in the Mediterranean, and the proliferation of aquaculture and marine infrastructures over the past decades, we anticipate the continued arrival of non-indigenous species, even those with low dispersal abilities such as *H. momus*. We

emphasize the need to employ an integrative approach such as that presented here in order to advance towards a holistic understanding of marine biological invasions.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.marpolbul.2012.06.029.

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