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Genetic structure of the long-snouted seahorse, *Hippocampus guttulatus*, in the Central–Western Mediterranean Sea

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The seahorse *Hippocampus guttulatus* reaches its highest abundance in confined environments, where it has unique biological and ecological traits that suggest significant genetic differentiation among populations. In the present study, we aimed to reveal the genetic structure of this species by analysing eight microsatellite loci and a mitochondrial DNA region (cytochrome b) of eight populations from the Central–Western Mediterranean Sea, including lagoon sites. Levels of genetic diversity, as measured by the total number of alleles, number of private alleles, allelic richness and heterozygosity, ranged from low to moderate. The overall value of inbreeding was high, indicating a deficiency in heterozygotes. The haplotype network had a star-like construction, with the most common haplotype present in all populations. Data from the two molecular markers congruently displayed a similar pattern and revealed low genetic differentiation, notwithstanding predictions based on species traits. The observed genetic structure is probably the result of both historical population demographic events and current gene flow. The investigated lagoons, however, revealed a unique genetic profile, which is especially highlighted by the Taranto population. At this site, the results also showed altered values of observed/expected heterozygosity and allelic richness, a characteristic of marginal populations. Our study suggests that lagoon populations should be managed as distinct genetic units.

ADDITIONAL KEYWORDS: bottleneck – conservation – dispersion – gene flow – microsatellites.

INTRODUCTION

The management and conservation strategies of endangered species require comprehensive knowledge of genetic diversity and the degree of connectivity

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among populations, which are important for determining species evolutionary potential across spatial and temporal scales (Frankham *et al.*, 2010; Cooke *et al.*, 2016). In the marine environment, genetic homogeneity is expected across vast areas due to the lack of obvious physical barriers to dispersal and the existence of planktonic larvae in many species (Cowen *et al.*, 2007). Although dispersal capacity is considered one of the principal factors in shaping population genetic structure, it may not always be the only driver of diversification (Rossi *et al.*, 2019). Indeed, population structure is often the result of a complex interaction between environmental, historical and individual or species-specific characteristics, including local adaptation (Gentili *et al.*, 2018), historical vicariance (Nascimento *et al.*, 2018), past bottleneck events (Shama *et al.*, 2011), oceanic currents (Rossi *et al.*, 2019), habitat discontinuities (Barber *et al.*, 2002), isolation by distance (Mims *et al.*, 2016), limited dispersal abilities (Ferreira *et al.*, 2015), behaviour and life history strategies (Nathan *et al.*, 2008).

Significant genetic differentiation among populations can be found in marine species with high dispersal potential (DeWoody & Avise, 2000), while other species may display genetic homogeneity despite predictions of the substantial population structure resulting from their biological and ecological traits, such as sedentary behaviour, monogamy and high site fidelity (e.g. Porrini *et al.*, 2015).

The European long-snouted seahorse *Hippocampus guttulatus* Cuvier, 1829, a relatively sedentary species that inhabits the North-Eastern Atlantic Ocean, the Mediterranean and the Black Sea (Lourie *et al.*, 1999, 2016), raises many conservation concerns because of the severe population declines in recent decades (Pollom, 2016). This has led to the species inclusion on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, in which it is listed as Data Deficient at a global level (Pollom, 2017), whereas in the Mediterranean basin and along the Italian coast, it is considered as Near Threatened (Pollom, 2016; Relini *et al.*, 2017). As with other congeneric species, the long-snouted seahorse is characterized by sedentary behaviour with low swimming capabilities, small home-ranges and high site fidelity (Curtis & Vincent, 2006). Furthermore, *H. guttulatus* exhibits high mate fidelity (Foster & Vincent, 2004; Woodall, 2009) and a short planktonic juvenile phase (Boisseau, 1967), while reproductive rates of adults are limited by low fecundity and small brood sizes. Although these particular traits may imply restricted migration and thus genetically structured populations, the genetic homogeneity of seahorse populations has been shown at large geographical scales (Woodall *et al.*, 2015; Riquet *et al.*, 2019a).

Hippocampus guttulatus can be found in different shallow coastal habitats, but it seems to reach the highest abundances in marine lagoons (Curtis & Vincent, 2005; Louisy, 2011; Caldwell & Vincent, 2012; Gristina *et al.*, 2015; Lazic *et al.*, 2018). These habitats, however, are frequently exposed to a wide range of environmental conditions, including changes in salinity and temperature (Gonzalez-Wanguemert *et al.*, 2006). Such variations, together with the typical isolation of confined environments, may exert strong selective pressures and thus could drive modifications of a species genetic pattern (e.g. Sanford & Morgan, 2011). Indeed, significant genetic differences between populations from coastal lagoons and the open sea have been established in many aquatic species (Allegrucci *et al.*, 1997; Gonzalez-Wanguemert *et al.*, 2006; Bisol *et al.*, 2007; Marko & Barr, 2007; Gonzalez-Wanguemert *et al.*, 2009). Past studies of the genetic structure of *H. guttulatus* have demonstrated the existence of four cryptic lineages across the entire species distributional range, where one of them is considered to be exclusive of the Mediterranean lagoons (Woodall *et al.*, 2015; Riquet *et al.*, 2019a). Along the Italian coast, the presence of multiple populations has only recently been highlighted, comprising the dense and important population in the marine lagoon of Taranto in southern Italy (Gristina *et al.*, 2015, 2017a; Lazic *et al.*, 2018). Demographic abundance values of this population are among the highest in the Mediterranean (Gristina *et al.*, 2015) and comparable to those of the Atlantic lagoons (Curtis & Vincent, 2005; Caldwell & Vincent, 2012). The present study addresses the question of a finer-scale genetic structure of threatened *H. guttulatus* in a poorly studied area, which with its numerous, and in the case of Taranto lagoon, dense seahorse populations could complement existing knowledge while providing valuable information for seahorse conservation. In the present study, a combination of microsatellite and mitochondrial (cytochrome b) markers was used to investigate the genetic structure and degree of differentiation of *H. guttulatus* in the Central–Western Mediterranean Sea with a particular emphasis on heterogeneous lagoon environments.

MATERIAL AND METHODS

Sample collection

A total of 119 *H. guttulatus* individuals were collected at eight locations in the Central–Western Mediterranean Sea (Fig. 1). Small pieces of skin filament tissue were removed *in situ* underwater with the non-lethal (Gristina *et al.*, 2017b) skin filament clipping procedure. After sample collection, all animals

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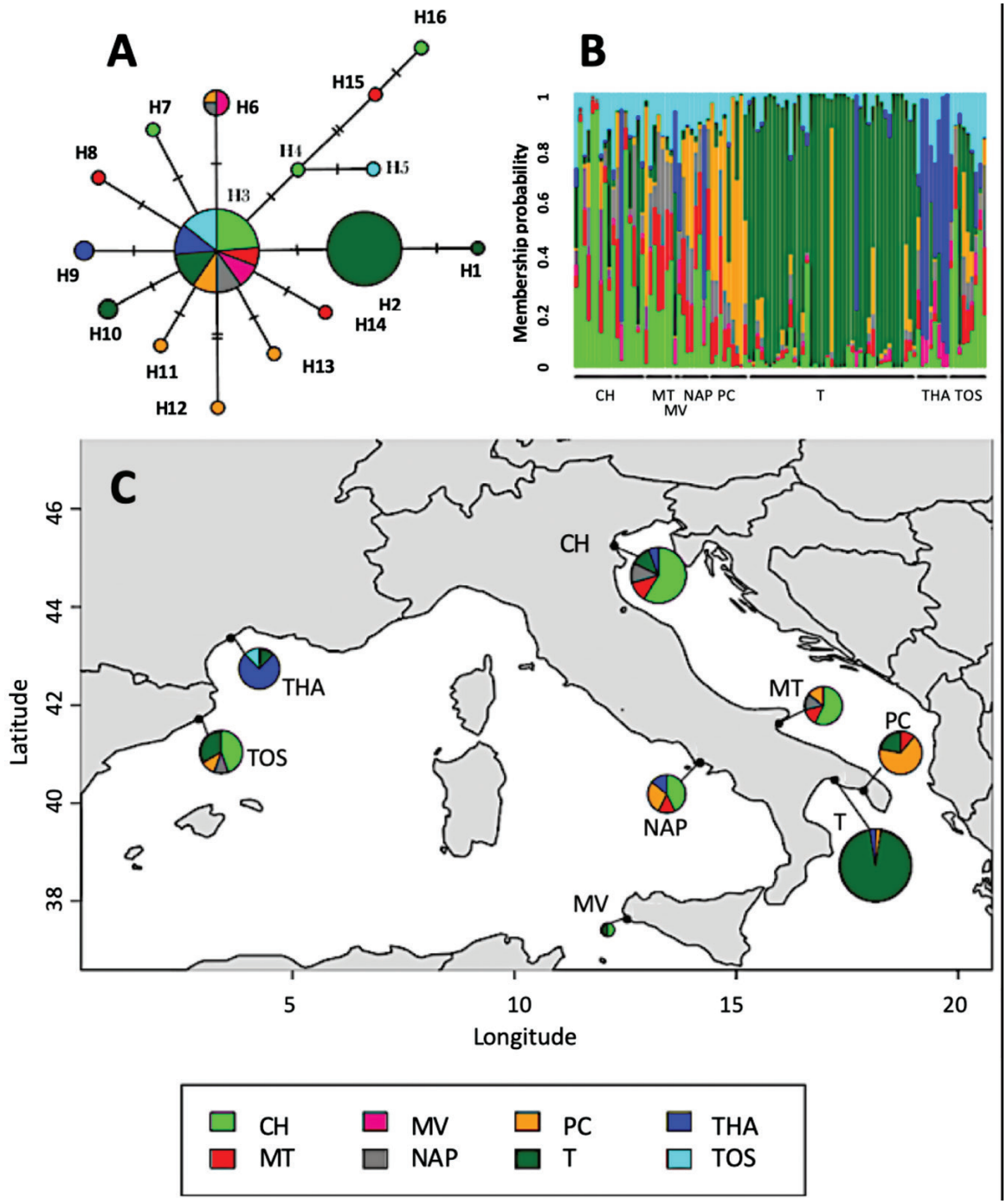

Figure 1. A, Analysis of *cytb*. Minimum spanning network for *cytb* haplotypes constructed from sequence data. B, microsatellite analysis. Population membership probability on the basis of their genotypic profiles according to DAPC. C, geographical distribution of microsatellite clusters. The pie diagrams show the frequency distribution of each cluster among populations: CH, Chioggia; MT, Mattinata; MV, Mazara del Vallo; NAP, Naples; PC, Porto Cesareo; T, Taranto; THA – Thau; and TOS, Tossa del Mar. An asterisk near the name of the sampling site indicates the lagoon site.

were released at the same point from which they were collected. All samples were preserved in 96% ethanol at 4 °C for subsequent genetic analysis.

CYTOCHROME B ANALYSIS

Total genomic DNA was extracted from skin filaments using the standard cetyltrimethylammonium bromide

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protocol (Doyle & Doyle, 1987). A fragment of the mitochondrial DNA (mtDNA) cytochrome b gene was amplified using the primers GUTTCYTB_F and GUTTCYTB_R (Woodall, 2009). The PCR conditions for cytochrome b were as follows: an initial denaturation for 2 min at 95 °C, followed by 35 cycles of 95 °C (30 s), 60 °C (30 s) and 72 °C (60 s), and a final extension at 72 °C for 10 min. PCR products were purified and sequenced by Macrogen (www.macrogen.com).

Electropherograms were checked using FinchTV (Geospiza, Inc., Seattle, WA, USA; http://www.geospiza.com) and minor changes were made by eye. A final consensus alignment was computed with MEGA 5.0 (Tamura *et al.*, 2011). After the final alignments were obtained, the number of haplotypes (n), and nucleotide (π) and haplotype (h) diversities for the entire dataset and across regions were estimated using DnaSP v.5.1 (Librado & Rozas, 2009). Finally, to infer gene-genealogies among *H. guttulatus* populations, a Minimum Spanning Network (MSN) was computed using the software PopART (Bandelt *et al.*, 1995).

Microsatellite analysis

All samples were amplified at 12 microsatellite loci (Pardo *et al.*, 2007). However, four microsatellite loci exhibited reaction inconsistency in most samples and were omitted from subsequent analyses. Thus, the final dataset consisted of eight genotyped microsatellite loci (Hgu-USC2, Hgu-USC4, Hgu-USC5, Hgu-USC7, Hgu-USC9, Hgu-USC11, Hgu-USC12, Hgu-USC13). Microsatellite primers were synthesized commercially, with the 5′ end of the forward primer labelled with one of the following fluorescent dyes: 6FAM, VIC, NED or PET (Applied Biosystems, Foster City, CA, USA). The following PCR amplification conditions were used: an initial denaturation for 3 min at 94 °C, followed by 35 cycles of 94 °C (30 s), 54–56 °C (30 s) and 72 °C (60 s), and a final extension at 72 °C for 10 min. PCR products were genotyped by Macrogen, using an ABI 3130xl Genetic Analyzer with the GS500 LIZ size standard control. Allele sizes were scored using the R package Fragman (Covarrubias-Pazaran *et al.*, 2016).

Allele frequencies, expected and observed heterozygosity (H_{exp} and H_{obs}), average number of alleles (A), number of private alleles (Np) and allelic richness (Ar) were estimated for each locus and sampling location using the R package hierfstat (Goudet, 2005). Deviations from Hardy–Weinberg equilibrium (HWE) were tested for each locus, pairs of loci and sampling location using the R package Pegas (Paradis, 2010). Sequential false discovery rate (FDR) correction for multiple tests was applied for HWE tests of significance because of the large number of tests involved (Benjamini & Hochberg, 1995). The occurrence of putative null alleles was evaluated using the R package PopGenReport (Adamack & Gruber, 2014).

Population structure was investigated by spatial principal component analysis (sPCA), which allows cryptic spatial patterns of genetic variability to be investigated. The sPCA yields scores summarizing genetic variability and spatial structure among individuals (or populations). Given genetic data and spatial coordinates, it maximizes the product of variance and spatial autocorrelation (Moran's I index), which allows for a distinction of global from local structures and random noise (Jombart, 2008). Spatial information in sPCA was modelled through a connection network based on the Delaunay triangulation criteria. Successively, population membership probability was evaluated using discriminant analysis of principal components (DAPC). sPCA scores were used in DAPC to evaluate a posteriori correct assignment of individuals to each sampled population.

Finally, Bayesian cluster analysis was performed using the software Structure 2.3.4. (Pritchard *et al.*, 2000) to detect the number of genetic clusters (K) and admixture within the dataset. Structure analysis allowed the search of a best cluster ranging from 2 to 8, assuming an 'admixture model' in which every individual has ancestry from one or more K genetically distinct sources. The Markov chain Monte Carlo (MCMC) search was performed using 100 000 repetitions after a burn-in (set to 10 000), replicated 10 times for each K value. CLUMPAK (Kopelman *et al.*, 2015) was used to post-process the Structure output to visualize the population structure of each K tested.

RESULTS

A 518-bp fragment was obtained from sequencing of the cytochrome b (*cytb*) gene. All *cytb* sequences were deposited at GenBank (accession numbers: MT276601, MT311875–MT311957, MT386084–MT386091). Overall haplotype diversity (Hd) was moderate, with an average value of 0.675 ± 0.035, while nucleotide diversity was low (π = 0.00226 ± 0.00029). Although haplotype diversity ranged from 0.286 ± 0.196 at Tossa del Mar to 0.800 ± 0.172 at Mattinata, most of the investigated populations displayed low values. Nucleotide diversity (π) ranged from 0.00083 ± 0.00049 at Naples to 0.00341 ± 0.00129 at Mattinata (Table 1).

A total of 16 haplotypes were found. The MSN presented a star-like pattern (Fig. 1A). For most cases, only a one-step mutation was found between the most common and the other haplotypes. The most common haplotype, H3, found in 42 individuals, occupied a central position and was shared by all populations. Haplotype H6 was shared by three populations, while other haplotypes were private to each population

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Table 1. Description of sampling sites and cytochrome b sequence diversity: sampling sites, site code, site description (open water/lagoon), number of samples used for mtDNA analysis (*N*), number of polymorphic sites (*Np*), number of haplotypes (*H*), haplotype (*Hd*) and nucleotide diversity (π) (SD, standard deviation)

Site	Site code	Site description	Latitude	Longitude	*N*	*Np*	*H*	*Hd* ± SD	π ± SD
Chioggia	CH	Open water	45°22′87.6″	12°30′53.6″	13	5	4	0.423 ± 0.164	0.00184 ± 0.00100
Mattinata	MT	Open water	41°73′15.4″	16°11′05.4″	6	5	4	0.800 ± 0.172	0.00341 ± 0.00129
Porto Cesareo	PC	Open water	40°25′68.9″	17°89′07.2″	8	5	5	0.786 ± 0.151	0.00262 ± 0.00081
Taranto	T	Lagoon	40°48′50.8″	17°26′02.1″	40	3	4	0.383 ± 0.088	0.00097 ± 0.00025
Mazara del Vallo	MV	Open water	37°64′22.2″	12°59′03.3″	5	1	2	0.400 ± 0.237	0.00090 ± 0.00053
Naples	NAP	Open water	40°82′57.6″	14°23′46.8″	7	1	2	0.400 ± 0.237	0.00083 ± 0.00049
Thau	THA	Lagoon	43°39′25.7″	3°60′32.8″	8	1	2	0.476 ± 0.171	0.00097 ± 0.00035
Tossa del Mar	TOS	Open water	41°71′89.2″	2°93′47.6″	7	2	2	0.286 ± 0.196	0.00117 ± 0.00080

(Supporting Information, Table S1). Haplotype H2, the largest exclusive haplotype found in the Taranto population, had 31 individuals (Fig. 1A; Table S1).

The microsatellite loci displayed low to moderate levels of genetic variability (Table 2). A total of 206 alleles over eight microsatellite loci were observed. Total number of private alleles was 22 (mean Pa = 2.75). The Taranto population had the highest number of private alleles (*Np* = 7), while Mattinata and Thau had none. Allelic richness across all populations was low (mean *Ar* = 1.39), ranging from 1.32 at Taranto to 1.42 at Chioggia, Mattinata and Tossa del Mar. The level of observed heterozygosity (Table 2) across all populations was low to moderate (mean H_{obs} = 0.35; range from 0.23 at Porto Cesareo to 0.44 at Mattinata and Mazara del Vallo), and lower than the expected heterozygosity (Table 3) (mean H_{exp} = 0.41; range from 0.3 at Porto Cesareo to 0.54 at Mazara del Vallo). The loci were in equilibrium in all populations, except for Hgu-USC7 and Hgu-USC13 that deviated significantly from HWE in the Taranto population (Supporting Information, Table S2). The inbreeding coefficient F_{is} displayed positive values in all populations (mean F_{is} = 0.16), ranging from 0.11 at Chioggia to 0.23 at Taranto (Table 3). The same coefficient also had positive values for at least one locus in all populations (Supporting Information, Table S3). A significant, but low, global F_{st} value (mean F_{st} = 0.04) was found. However, pairwise F_{st} values ranged from 0.030 to 0.11 (Table S4).

The sPCA scatterplot, based on the first two spatial principal components (λ_1 and λ_2; Fig. 2A,B), explained an important fraction of the variance and spatial autocorrelation and clearly discriminated against three main groups. The Taranto population appeared as the most separated. Populations from Naples, Chioggia and Mattinata largely overlapped and constituted a distinct group, whereas Tossa del Mar, Mazara del Vallo, Porto Cesareo and Thau formed the third population group. The Thau population showed a certain degree of separation on λ_2 and λ_3 (Fig. 2C). A posteriori attribution by DAPC showed that only individuals from Taranto and Thau demonstrate a high percentage of attribution of individuals based on their genotyping profile (Fig. 1B, C).

Results from sPCA and DAPC (Figs 1B, C, 2) were in agreement with the Bayesian clustering (Fig. 3; Supporting Information, Fig. S1), which identified the same clusters at *K* = 3. Taranto demonstrated low admixture of a few individuals that appeared genetically closer to the Western Mediterranean cluster. At *K* = 4, Thau and Tossa de Mar were split into two separate clusters. Taranto showed a certain level of admixture with Thau, but still maintained a clear genetic separation. With increasing *K*, Taranto was the only population that maintained a clear distinction and lower admixture level (Fig. S2).

DISCUSSION

The present study provides insights into the genetic structure and diversity of *H. guttulatus* in the Central–Western Mediterranean part of the species range while providing a further step towards our understanding of genetic differentiation in lagoon populations. In accordance with previous studies on large spatial scales (Woodall *et al.*, 2015; Riquet *et al.*, 2019a),

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Table 2. Genetic diversity indices for eight microsatellites loci at sampling sites: total number of alleles (N_A), number of private alleles (*Np*), allelic richness (*Ar*), observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp})

Locus		Chioggia	Mattinata	Mazara del Vallo	Naples	Porto Cesareo	Taranto	Thau	Tossa del Mar
Hgu-USC2	N_A	3	3	2	2	2	3	2	2
	Np	1	0	0	0	0	0	0	0
	Ar	1.51	1.46	1.43	1.5	1.46	1.33	1.44	1.35
	H_{obs}	0.71	0.57	0.50	0.29	0.22	0.20	0.38	0.43
	H_{exp}	0.52	0.46	0.50	0.55	0.49	0.33	0.46	0.36
Hgu-USC4	N_A	5	3	3	2	2	3	3	4
	Np	0	0	0	0	0	1	0	0
	Ar	1.32	1.56	1.71	1.35	1.23	1.43	1.55	1.53
	H_{obs}	0.35	0.86	1.00	0.43	0.25	0.44	0.50	0.33
	H_{exp}	0.32	0.56	0.75	0.36	0.23	0.43	0.57	0.56
Hgu-USC5	N_A	4	4	1	4	3	4	2	4
	Np	1	0	0	0	0	0	0	1
	Ar	1.47	1.55	1	1.55	1.33	1.27	1.29	1.65
	H_{obs}	0.24	0.43	0	0.57	0.38	0.30	0.33	0.67
	H_{exp}	0.48	0.58	N_A	0.57	0.34	0.27	0.30	0.67
Hgu-USC7	N_A	13	8	2	6	6	11	7	8
	Np	3	0	2	0	0	3	0	2
	Ar	1.88	1.84	1.67	1.79	1.74	1.78	1.82	1.84
	H_{obs}	0.82	0.71	1	0.57	0.56	0.59	0.75	0.67
	H_{exp}	0.90	0.88	N_A	0.85	0.78	0.79	0.86	0.88
Hgu-USC9	N_A	3	3	2	4	3	3	3	4
	Np	0	0	1	1	1	1	0	0
	Ar	1.39	1.30	1.57	1.38	1.21	1.07	1.42	1.39
	H_{obs}	0.44	0.17	0	0.29	0.22	0.08	0.25	0.22
	H_{exp}	0.40	0.33	1	0.40	0.22	0.08	0.45	0.41
Hgu-USC11	N_A	2	2	1	2	1	2	1	2
	Np	0	0	0	0	0	1	0	0
	Ar	1.48	1.25	1.00	1.25	1	1.43	1	1.30
	H_{obs}	0.29	0.29	0	0.29	0	0.32	0	0.11
	H_{exp}	0.49	0.26	0	0.26	0	0.43	0	0.32
Hgu-USC12	N_A	2	2	2	2	3	3	3	3
	Np	0	0	0	0	1	0	0	0
	Ar	1.21	1.16	1.43	1.14	1.44	1.07	1.49	1.30
	H_{obs}	0.12	0.17	0.50	0.14	0.11	0.02	0.38	0.33
	H_{exp}	0.22	0.17	0.50	0.14	0.47	0.07	0.52	0.31
Hgu-USC13	N_A	3	2	2	2	2	4	1	1
	Np	1	0	0	0	0	1	0	0
	Ar	1.11	1.25	1.43	1.25	1.12	1.18	1	1
	H_{obs}	0.12	0.29	0.50	0.29	0.12	0.10	0	0
	H_{exp}	0.12	0.26	0.50	0.26	0.12	0.19	0	0

the results suggest homogeneity of seahorse populations, with the exception of the unique genetic profiles of lagoons.

The overall value of genetic diversity across all populations, for microsatellite loci, was low (global H_{obs} = 0.35). An explanation can be found in some characteristics of the seahorses, such as monogamy, sedentary behaviour and high site fidelity, linking genetic diversity to behaviour. However, previous studies have reported higher values in other populations of *H. guttulatus* (Pardo *et al.*, 2007), as well as in other congeneric species (e.g. *H. abdominalis* in Nickel & Cursons, 2012; *H. hippocampus* in Lòpez et al., 2010; *H. capensis* in Galbusera *et al.*, 2007). Low observed heterozygosity points to high heterozygosity deficiency and high levels of inbreeding (global F_{is} = 0.16). Inbreeding is predicted to be more problematic in small populations where closely related individuals

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Table 3. Total number of individuals (n) used for microsatellite analysis, expected (H_{exp}) heterozygosity and coefficient of inbreeding (F_{is}) for each sampled population

SITE	n	H_{exp}	F_{is}
Chioggia	17	0.46	0.11
Mattinata	7	0.45	0.003
Mazara del Vallo	2	0.54	0.13
Naples	7	0.39	0.09
Porto Cesareo	9	0.3	0.2
Taranto	41	0.31	0.23
Thau	8	0.39	0.17
Tossa del Mar	9	0.45	0.21

are more likely to breed together (Lienert, 2004) and can cause a decrease in abundance (Frankham, 2003). This is a possible scenario for *H. guttulatus* which, in the past, was a common species along the Italian coast, but is now declining (Lazic *et al.*, 2018).

Observed heterozygosity was lower than expected (H_{exp} = 0.41), with a departure from HWE. Significant deviations from HWE were observed at two loci in one population (Taranto). Heterozygosity deficiency observed at these loci can have various causes (Rosewich *et al.*, 1999), and although distinguishing among them is difficult (Christiansen & Frydenberg, 1974), the most likely explanation involves inbreeding and the presence of null alleles in one of the loci.

Both mitochondrial and microsatellite markers congruently showed a similar genetic pattern, revealing overall low genetic structuring among *H. guttulatus* populations, in accordance with previous observations on other populations of the same species (Lopez *et al.*, 2015; Woodall *et al.*, 2015; Riquet *et al.*, 2019a), but also on sympatric *H. hippocampus* (Woodall *et al.*, 2011). Low levels of differentiation could indicate the existence of gene flow among populations. If so, it could be caused by the dispersion of juveniles in the first few weeks of their life while still part of the pelagic zooplankton (Boisseau, 1967; Curtis & Vincent, 2006; Morgan & Vincent, 2007), although occasional long-distance dispersal of adults, autonomously or by rafting, is also possible (Lourie *et al.*, 2005; Teske *et al.*, 2007; Luzzatto *et al.*, 2013). Another explanation for the observed shallow genetic structure includes insufficient time elapsed for the occurrence of the genetic signature after a bottleneck during the Pleistocene and affected all Mediterranean populations. Bottleneck events commonly lead to a decrease in genetic diversity as a result of population size reduction (Landergott *et al.*, 2001). During the Pleistocene glacial periods, *H. guttulatus* contracted to at least one refugial population, after which the species again expanded, although the recolonization process was influenced by oceanic currents and the

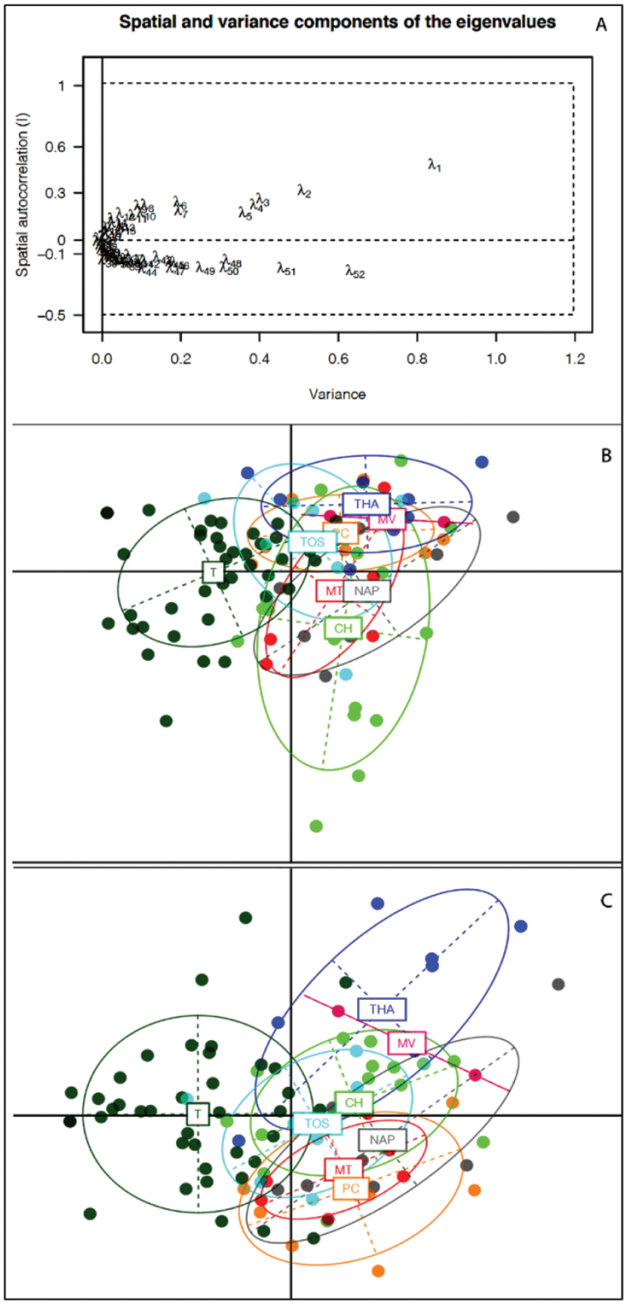

Figure 2. Spatial principal component analysis (sPCA). A, variance and spatial autocorrelation explained by each sPCA axis. B, sPCA plot based on λ_1 (horizontal) and λ_2 (vertical). C, sPCA plot based on λ_1 (horizontal) and λ_3 (vertical).

species' low dispersal potential (Woodall *et al.*, 2015). The hypothesis of historical dispersal events among populations, followed by population expansion, is consistent with the analysis of *cytb* sequences. In fact, mitochondrial data indicate that the species exhibits a star-like phylogeny, with a common ancestral haplotype

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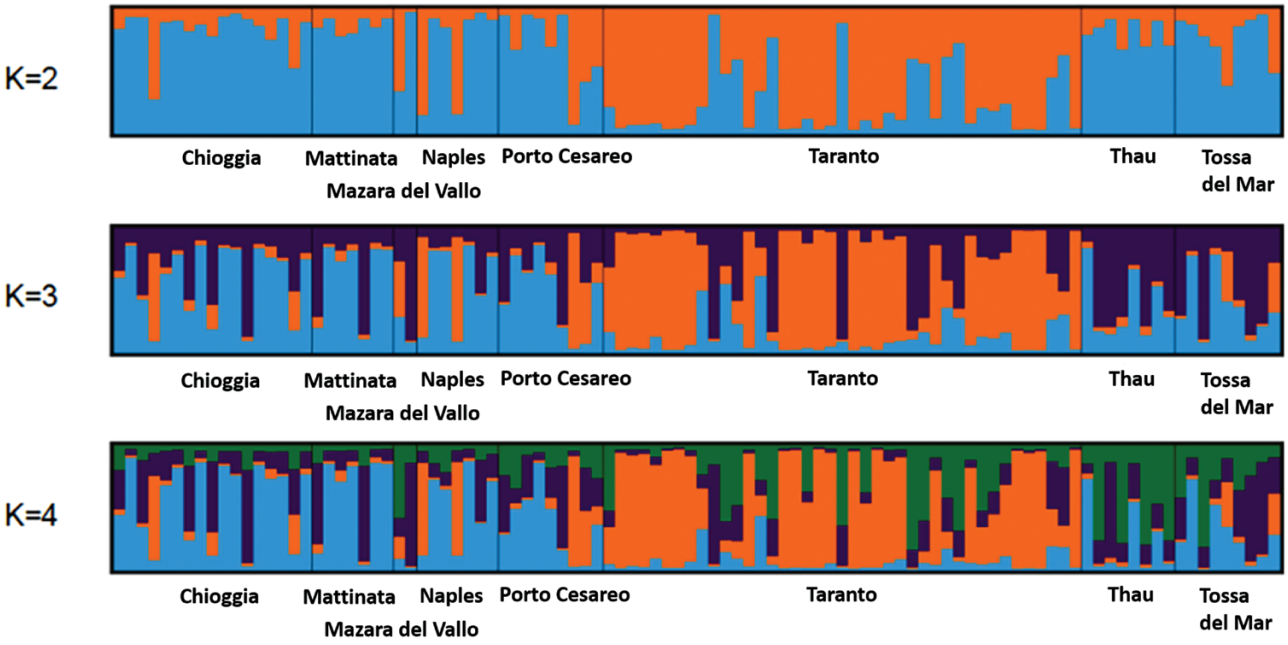

Figure 3. Bayesian cluster analysis (from $K = 2$ to $K = 4$) using data from eight microsatellite loci.

that radiated to numerous closely related haplotypes, as already observed in many other marine fishes (e.g. Aboim *et al.*, 2005; D'Amato & Carvalho, 2005). Low differentiation among populations has also been suggested by nuclear markers. Despite the occurrence of significant genetic differentiation between some population pairs, F_{st} values were generally low, and in half of the compared population pairs, the F_{st} value was lower than 0.05 (Supporting Information, Table S4). However, sPCA (Fig. 2) indicated the existence of three population groups. The Taranto population formed a separate group, while the rest of the Italian populations were divided into two genetic groups that were not fully consistent with their geographical distribution. Thau, another Mediterranean lagoon, also exhibited a certain degree of differentiation.

DAPC suggested that the genotypic profile of Taranto and Thau, followed by Porto Cesareo and Chioggia, have good discriminatory power, allowing the correct reclassification of many individuals, whereas the genotypic profile of other populations was not sufficiently diagnostic for a good percentage of correct reclassification (Fig. 1B). This result was fully congruent with Bayesian clustering (Fig. 3), which depicted the identical scenario. According to this analysis, Taranto and Thau were the most differentiated populations, while Taranto had the lowest level of admixture. Thus, all analyses congruently demonstrated the lack of a strong genetic structure, but highlighted the occurrence of unique genetic profiles in the Mediterranean lagoons, in agreement with a previous study (Riquet *et al.*, 2019a).

Lagoon environments are considered potential sites for the emergence of different genetic constituencies because they can cause genetic divergence among populations, as already observed in several sedentary species of invertebrates (Gonzalez-Wanguemert *et al.*, 2009; Vergara-Chen *et al.*, 2010; Marino *et al.*, 2010). The genetic divergence of seahorse populations in lagoons may result from variable influences of both evolutionary and environmental factors. Indeed, geographical barriers and particular environmental conditions of lagoon systems might hinder dispersal mechanisms (Vergara-Chen *et al.*, 2010) , that together with high site fidelity and small brood size of seahorses could promote the population genetic differentiation. Lagoons are characterized by variability of physical and chemical parameters, and in fact, it has been hypothesized that populations exposed to wide environmental fluctuations in temperature and salinity (Veliz *et al.*, 2004) may differentiate due to genetic drift or natural selection (Cimmaruta *et al.*, 2003). Indeed, lagoons are frequently exposed to heavy bottlenecks and strong evolutionary pressures (Bamber & Henderson, 1985). For the Taranto population, in particular, the dominance of exclusive haplotypes, as well as low haplotype and nucleotide diversities, could indicate that the population has passed through a severe or long bottleneck. Although seahorses can survive extreme environmental conditions (Teske *et al.*, 2003), the relatively low genetic diversity of *H. guttulatus* in heterogeneous lagoon systems should be considered as indicating the extinction risk in a threatened species. Nevertheless, a recent study,

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mostly in agreement with the present data, suggested the Thau *H. guttulatus* population was in a good genetic state (Riquet *et al.*, 2019b). By contrast, the most separated Taranto population has characteristics of a distressed marginal population, including low levels of expected/observed heterozygosity and allelic richness, reduced genetic diversity, and altered phenotypic and life-history traits (Michalski & Durka, 2007; Lazic *et al.*, 2018).

Seahorses are considered as flagship species of conservation efforts, and while numerous and abundant in the past, populations are now in decline (Lazic *et al.*, 2018). The present study has demonstrated a shallow genetic structure of *H. guttulatus*, probably as the result of both population demographic events and current gene flow. The study has also demonstrated that more isolated populations of *H. guttulatus* are likely to have a particular genetic structure not shared with those from the rest of the basin. This is particularly relevant for the Taranto lagoon seahorses, whose private alleles and genotypes, together with a high density of individuals, may represent a significant proportion of the species diversity. Nonetheless, not just at this site (Lazic *et al.*, 2018), but also at many other sites of *H. guttulatus* occurrence, there are no particular measures for the protection of this species (Pollom, 2017). This study suggests that specific regional and international initiatives should be put in place to protect the species, perhaps in the form of a network of protected areas (Woodall *et al.*, 2018), whereas lagoons should be considered as separate genetic units. Although lagoons do not seem to contribute to the genetic diversity within the basin, these particular environments, with their suitable habitats and rich seahorse populations (Curtis & Vincent, 2005; Louisy, 2011; Caldwell & Vincent, 2012; Gristina *et al.*, 2015; Lazic *et al.*, 2018; Ape et al., 2019), need to be protected because they may be important for maintaining the diversity of *H. guttulatus* throughout its distribution range.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Distribution of haplotypes within samples used in the present study.
Table S2. Hardy–Weinberg equilibrium *P*-test.
Table S3. F_{is} coefficients.
Table S4. F_{st} values calculated for each pair of sampling sites.
Figure S1. Bayesian cluster analysis.
Figure S2. Bayesian cluster analysis (from $K = 2$ to $K = 8$) using data from eight microsatellite loci.

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