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**Past and present drivers of population structure in a small coastal fish, the European long snouted
seahorse *Hippocampus guttulatus***

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Abstract

The effective design of species conservation and management programs is reliant on information such as extant geographic distribution, taxon-specific life-history characteristics, and the relative influence of historic processes and contemporary environmental parameters in shaping population genetic diversity. Seahorses are small coastal fish, weak swimmers as adults and with brooded young, limiting their dispersal potential. Seahorses live in sheltered locations, including estuaries which are physically isolated from each other. Therefore panmixia across their geographic range is unlikely. *Hippocampus guttulatus*, a seahorse inhabiting European waters, has a geographic range spanning a number of contemporary oceanographic features that are proposed barriers to gene flow. Thus this fish is well-placed to test the relative contributions of environment and life-history factors in shaping contemporary population structuring.

This study found that mitochondrial DNA and nuclear DNA (microsatellite) genotype data are concordant in suggesting that, like many other small fishes in European waters, *H. guttulatus* extant populations expanded from at least one southern European refugial population. Subsequent population differentiation of four geographic lineages reflects contemporary oceanographic barriers to gene flow. Demographic analyses suggest a northward expansion from a southern refugium, and long-term isolation between Black Sea and Mediterranean Sea populations. Moreover *H. guttulatus* contemporary population distribution and population structure is predominately explained by historic and oceanographic influences, rather than life-history traits and associated habitat preference. These findings suggest that conservation of genetic diversity in *H. guttulatus* may be aided by a network of Marine Protected Areas (MPAs), implemented to conserve coastal species and habitats, but the species' unusual life history and gamete retaining behaviours should be considered as part of management decisions including MPA design and fisheries management plans.

Keywords: *Hippocampus guttulatus*, conservation genetics, phylogeography, Europe, seahorse.

Introduction

Marine conservation can be enhanced when species-specific biological, ecological and genetic data are considered in conjunction with environmental parameters. For example, estimations of genetic connectivity can help resolve the relative influence of historical versus current environmental features and processes on contemporary population structuring. In marine species, many examples have shown the utility of genetic data in establishing the importance of life-history traits (Galindo et al. 2010), environmental factors (e.g. hydrodynamics Schunter et al. 2011) and historic processes (Maggs et al. 2008; Patarnello et al. 2007) in driving contemporary population diversity and structuring. Such studies have been used to inform species management and conservation strategies (Planes et al. 2009).

Marine species have been predicted to have little genetic structure due to propagule dispersing factors such as long larval phases and dispersal by oceanographic currents (Ward, et al. 1994). Whilst some species show close fit to expected patterns of high genetic connectivity (e.g. the coral *Astroides calycularis*, Casado-Amezua et al. 2012), many species that have potential for high gene flow do not exhibit panmictic populations (e.g. European anchovy, Zarraonaindia et al. 2012; bluefin tuna, Riccioni et al. 2010), and some species predicted to have substantial population substructure such as the marbled goby, a lagoon dwelling fish, show widespread genetic homogeneity (Merjri et al. 2011). Thus the population structures of marine species are determined by a complex interaction of factors, and may or may not be easily predicted.

Environmental factors are regularly shown to influence genetic differentiation of marine species. Factors include coastal topography (Nicastro, et al. 2008), oceanic currents (Quinteiro, et al. 2007), bathymetric profile (Hoarau, et al. 2002), habitat availability (Astolfi, et al. 2005), and temperature and salinity discontinuities (Jorgensen, et al. 2005). For example, water temperature changes seasonally in temperate zones, but currents (e.g. Gulf Stream) or depth (e.g. thermoclines) can also maintain distinct long-term temperature discontinuities. Historic events such as bottlenecks in population size (Bargelloni, et al. 2005), species range expansion (Wilson 2006) and vicariance (Arnaud-Haond, et al. 2007) occur in response to environmental or anthropogenic factors, and may also influence contemporary population connectivity, species geographic range, and the distribution of genetic diversity.

Both historic and extant environmental conditions in European waters of the NE Atlantic Ocean, Mediterranean Sea and Black Sea are known to have influenced their different faunal compositions. Within and between these waters there are a number of recognised barriers to individual dispersal that define population

divergence of marine species, such as the Straits of Sicily and the Almeria-Oran frontal system in the Mediterranean Sea (Galarza et al. 2009; Patarnello et al. 2007; Schunter et al. 2011).

Seahorses have distinct life-history characteristics that have led researchers to hypothesize limited connectivity among patchily distributed populations, as well as making them more vulnerable to habitat destruction and overexploitation (reviewed by Vincent et al. 2011). Predictions of low individual dispersal capacity result from traits such as internal fertilisation and brooding of young, a short planktonic juvenile phase (Boisseau 1967), and adult site fidelity, small home range (Curtis & Vincent 2006) and weak swimming ability (Blake 1976). However seahorses are known to move up to 150m daily, within a lagoon system (Caldwell & Vincent 2013) and have the potential for occasional migration events by rafting (Luzzatto et al. 2013; Perante et al. 2002; Vandendriessche et al. 2005). In addition, most seahorse species are socially (Foster & Vincent 2004) and genetically serially monogamous (Woodall et al. 2011a), which could result in a lower effective population size due to limited parental crossings. Seahorses therefore exhibit characteristics that suggest highly structured genetic populations.

Hippocampus guttulatus, Cuvier 1829, is distributed along coasts of the North-East Atlantic from the English Channel in the north to NW Africa in the south, and throughout the Mediterranean Sea and Black Sea (Lourie et al. 2004). Like many seahorses, *H. guttulatus* is a shallow coastal and estuarine dweller, often associated with seagrass beds (Curtis & Vincent 2005), potentially limiting dispersal across deep open water. Throughout its range these habitats are disjunct (Green & Short 2003) and as such this species exhibits non-continuous populations, which can reduce the chance of nearby populations mixing. Seahorses such as *H. guttulatus* are thus well placed to elucidate the relative influence of life history traits versus environmental and historical climatic factors in determining population connectivity.

A single recent study has assessed genetic variation in *H. guttulatus* across a small part of the species' geographic range (NW Iberian Peninsula), and found no significant barriers to gene flow (Lopez et al. 2015), but studies of three other syngnathid species from areas more representative of the geographic range of *H. guttulatus* suggest more defined population differentiation in such species. Contemporary population structure of the pipefish *Syngnathus typhle* has been shown to be influenced by Pleistocene glaciations, with post glacial recolonisation effects evident in movement of the geographic range north and eastwards (Wilson & Eigenmann Veraguth 2010), whilst another pipefish (*S. abaster*) displays significant post-glacial fragmentation and differentiation (Sanna et al. 2013). Similarly, both contemporary (i.e. oceanographic barriers) and historic factors (i.e. Pleistocene glaciation) were identified as shaping the population structure across European waters in

Hippocampus hippocampus, with no evidence for the limited propagule connectivity expected in this family (Woodall et al. 2011b).

In this study mtDNA and nuclear DNA (microsatellite) markers were applied to samples from the entire geographic range of *H. guttulatus* to: investigate contemporary genetic population structure; identify potential barriers to gene flow; infer demographic history, including times of population divergence and range expansion; and propose conservation and management practices in the light of data from this and other European seahorse species.

Materials and Methods

Sample acquisition and DNA extraction

Specimens were collected from 17 locations across the NE Atlantic Ocean, northern Mediterranean Sea and Black Sea, covering over 6000 km of coastline and with a range of 60-1200 km between neighbouring sites (Fig 1, Table 1). Seahorses generally live in low densities, are cryptic and are not commercially targeted in Europe, so they are particularly difficult to sample. As a result at some sites it was necessary to re-sample over successive days and/or consecutive years (site MSP). Tissue samples were collected from each individual *in situ* underwater and non-lethally to minimise impacts on individuals and populations (see Woodall et al. 2012). Genomic DNA was isolated from 3-4 mm² of dorsal fin tissue using a standard cetyltrimethyl ammonium bromide (CTAB) chloroform/isoamyl alcohol DNA extraction method (after Winnepenninckx, et al. 1993).

Mitochondrial DNA sequencing

Fragments of mitochondrial DNA in the hypervariable 5' end of the Control Region (CR) and the cytochrome b gene (*cytb*) were amplified for a maximum of 29 specimens from each of the 17 range-wide locations (Table 1). The CR was amplified using seahorse-specific primer HCAL2 (Teske et al. 2003) and *H. guttulatus*-specific primer HIPPCONR (5'AAG CCG AGC GTT CTC TCC 3'). The *cytb* was amplified using primer SHORSE 5.3L (Casey et al. 2004) and *H. guttulatus*-specific primer GUTTCYTB-R (5' AGG GGG TTC TAC AGG CAT TAC 3'). Each 50µl PCR reaction contained: 5µl 10X manufacturer provided buffer, 2.5µl MgCl₂ (50mM - Bioline, UK), 5µl deoxynucleotide triphosphate mix (dNTP) (1.25mM), 1.2 µl of each primer (10µM), 0.25µl Taq polymerase (5U/µl - Bioline, UK), 14.25µl H₂O and 20µl template DNA (10-50 ng). The PCR profile was composed of an initial denaturation step (2 min at 94 °C), followed by 35 cycles of denaturation (30 s at 94 °C), annealing (30s at 50 °C) and extension (60s at 72 °C), and a final extension step (2 min at 72 °C).

Amplified products were purified, using either PCR purification kit (Qiagen) or Exonuclease 1-Shrimp Alkaline phosphatase protocols, sequenced in both directions by Macrogen (Korea), then deposited in Genbank (Accession numbers: KM061952 to KM062016).

Amplification and screening of microsatellites

Twenty-five previously developed seahorse-specific microsatellite primers (Galbusera et al. 2007; Pardo et al. 2006) were tested for amplification, allelic variation, null alleles and stutter bands. Five polymorphic microsatellite loci were selected for final screening (Hgu4, Hgu12, Hcap11, Hcap25 & Hcap27); the other loci either failed to amplify or were monomorphic. In total 313 specimens were genotyped from ten locations for a minimum of 15 individuals per site (Table 2). Loci were amplified separately in 10 µl reactions containing 2 µl template DNA (1–5 ng), 1 µl manufacturer-provided buffer, 0.6 µl MgCl² (50 mM-Bioline), 1 µl dNTP mix (1.25 mM), 0.25 µl of each primer (10 µM, one being Cy5' labelled), 0.05 µl Taq polymerase (5U/µl-Bioline). The thermocycle profile comprised an initial denaturing step (3 min at 95°C), followed by 35 cycles of denaturing (30 s at 95°C), annealing (30 s at 50°C (Hgu12), 53°C (Hcap11 and Hcap25) or 55°C (Hgu4 and Hcap27), and extension (30 s at 72°C), with a final extension step (3 min at 72°C)). PCR products were run on 6% denaturing polyacrylamide gels in an ALFexpressII automated DNA sequencer (Amersham Pharmacia) and allele sizes scored using Fragment Manager v2.9 (Amersham Pharmacia).

Genetic Diversity

Sequence chromatographs were manually checked for errors and edited unambiguously in BIOEDIT v7.2.5 (Hall 1999), then consensus sequences were aligned using CLUSTAL X (Thompson et al. 1997). Genetic diversity indices of haplotype diversity (h) and nucleotide diversity (π) were calculated in ARLEQUIN v3.5.1.3 (Excoffier & Lischer 2010). Diversity was calculated for all sample locations and regions with a sample size of 15 or larger. Genealogy networks were used to visualise nucleotide sequence divergence and genetic relationships between haplotypes; implemented in TCS v1.21 (Clement et al. 2000).

Microsatellite locus number of alleles, conformity with Hardy Weinberg (HW) expectation and linkage disequilibrium were computed in GENEPOP v4.0 (Rousset 2008). Observed and expected heterozygosity were calculated in ARLEQUIN. Due to small samples sizes sites CGR and KGR were pooled to form a single Greek sample (GRE), after testing for allele frequency conformity between the individual samples.

Power Analysis

No evidence of null alleles was detected within any microsatellite locus using FreeNA (Chapuis & Estoup 2007), and a <1% genotyping error was established by re-scoring five separate gels of each locus and comparing

allele sizes with the original scoring. POWSIM v4.0 (Ryman and Palm 2006) was used to test the power of the microsatellite data to detect signals of genetic differentiation with current sample sizes, using different levels of genetic divergence ranging from $F_{ST} = 0.005$ to 0.200.

Genetic differentiation

Populations were combined into seven regions (Table 1) for testing for differentiation, based on geographic distance between sites and biogeographic provinces. Genetic structuring was assessed using AMOVA conducted in ARLEQUIN using Φ_{ST} (mtDNA) and F_{ST} (microsatellites) to test for significant differences within and between regions across Europe. To determine which pairwise comparisons contributed to the genetic structure inferred in AMOVA, two measures of genetic differentiation were used, the fixation index F_{ST} implemented in ARLEQUIN for mtDNA and FSTAT v2.9.2.3 (Goudet 2001) for microsatellites. Estimates of gene flow between regions were made using the maximum likelihood method (ML), implemented in MIGRATE 3.2.1 (Beerli & Felsenstein 2001). The estimates in MIGRATE were based on MCMC simulations using ten long chains and five short chains, of 150,000 and 11,250 genealogies respectively, with a burn-in of an additional 10,000, data recorded every 20 reconstructed genealogies. The mutation model was derived by calculating the gamma distribution (alpha) in PAUP* v4.0b10 (Swofford 2003).

Subpopulation assignment tests were performed on population level microsatellite data in STRUCTURE v2.3.4 (Pritchard et al. 2000) using both admixture and no admixture models with a burn-in of 5×10^5 and 1×10^6 MCMC chains. Both models were tested as some regions contained near-by unsampled populations, and some populations were close whereas some were isolated (SPORT and BISCAY), and these are more likely to have admixture than geographically distant/isolated ones (i.e. WMED and BLACK). All possible numbers of populations (K) were tested (1-9), using 20 replicates, and the most parsimonious were assessed according to ΔK (Evanno et al. 2005) using STRUCTURE HARVESTER web v0.6.92 (Earl & vonHoldt 2012).

The mantel test, which tests the correlation between genetic and geographic distance, was implemented in IBDWS v 3.23 (Jensen et al. 2005) using 30,000 randomisations on concatenated sequences and microsatellite genotypes separately. Distances between sampled sites were calculated using minimum sea distances (Table S1).

Historical Processes

To infer the probability of demographic parameters we used an approximate Bayesian computation (ABC) approach in the program DIYABC v2.03 (Cornuet et al. 2010; Cornuet et al. 2008), wherein molecular data are condensed into summary statistics and then compared to simulated data using a coalescent population model. For our model, we simulated four major regions of *H. guttulatus* distribution: 1) UK+BISCAY, 2) SPORT+MSP,

3) WMED+EMED, and 4) BLACK. This regional grouping was selected based on concordance of population differentiation estimates from both mtDNA and microsatellite analyses (see previous methods and Table 3 as well as Fig. 3 and 4) and inferred oceanographic regions. The posterior distributions of parameters were calculated based on 1 million simulations using a total of 48 summary statistics. The fit of summary statistics to the model and chosen prior distributions were evaluated by locating the observed value and each summary statistic within a principal component analysis of 5000 simulated data sets. Microsatellite summary statistics included Mean size variance, Two-sample F_{ST} , and $(du)^2$. Mitochondrial summary statistics included Mean pairwise differences, Variance of pairwise differences, Tajima's D, Private segregating sites, and Mean numbers of rarest segregating sites. Between-population statistics included Mean of pairwise differences and F_{ST} (Hudson et al. 1992). Simulations were based on a complete dataset of 214 individuals. Mutation rates for mtDNA and microsatellites were uniformly distributed with an upper and lower bound of $8.00E-9$ to $1.3E-8$ and $1.00E-005$ to $1.00E-004$ (in units of per site / per generation / per lineage) respectively. Uniform priors for effective population size ranged from (N_e) of 10×10^2 to 15×10^6 , and divergence time 10×10^2 to 10×10^5 scaled to a generation time of 1 year. Euclidean distances between the observed and simulated data set were computed using a local linear regression, and 5,000 of the closest simulated to the observed datasets were retained to estimate posterior distributions of 18 parameters, which included divergence times, effective populations sizes, and timing and magnitude of size change within each region (Table 4) (Beaumont et al. 2002; Cornuet et al. 2008).

Results

Population description

A total of 236 individuals were genotyped for both CR and *cytb* and concatenated to give a sequence of 991bp. The concatenated sequences revealed 70 haplotypes, with the most common haplotype seen in 28% of individuals across all regions. Total haplotype diversity was high ($h=0.91$) and nucleotide diversity was low ($\pi=0.003$) (Table 1). High haplotype diversity was found across all locations and regions, with the exception of the UK (PUK) and southern France (SFR). Nucleotide diversity was low across all populations and regions. The maximum parsimony network of concatenated sequences resembles a shallow star-like pattern (Fig. 2). Little geographic structuring can be seen in the network; the most common haplotype is represented in all regions; almost all other common haplotypes are found in multiple regions, except for the Black Sea; all regions display multiple private haplotypes. However the percentage of private haplotypes present differed considerably between regions; the UK has none and the Black Sea 81%, whereas the other regions have between 40-55%.

All 313 individuals sampled were genotyped at five microsatellite loci, with all loci displaying no significant overall departures from Hardy Weinberg expectations of genotype frequencies or linkage disequilibrium. Moderate to low levels of genetic variability were observed at all loci (Table 2), but private alleles were present at each locus and all sampled locations. Observed and expected heterozygosity (Table 2) did not display geographic patterns, and no indication of inbreeding (F_{IS}) were significant following Bonferroni correction. Power analysis based on sample size and screened microsatellite loci suggested that genetic divergence can be detected with >93% confidence for F_{ST} of 0.005, 98% confidence for F_{ST} of 0.007 and > 99.9% confidence for $F_{ST} \geq 0.010$. An expected F_{ST} of zero estimates α to be 0.060–0.078, indicating expected levels of type I error. These results suggest that the five loci have the power to detect low levels of genetic differentiation (down to F_{ST} of 0.005).

Genetic differentiation

The global F_{ST} (Φ_{ST} 0.089 $p < 0.0001$ mtDNA; $F_{ST} = 0.087$, $p < 0.0001$ microsatellites) indicated that there was significant population genetic differentiation across the sampled range. The AMOVA indicated that the greatest proportion of variation at both mtDNA and nDNA loci is among individuals within sample sites, although both marker types detected significant variation among regions (mtDNA: 10.4%, Φ_{SC} 0.104, $p < 0.0001$) (nDNA; 7.28%, F_{SC} 0.157, $p < 0.005$), with marginally significant variation between locations within regions for the microsatellite data (Table S2).

When samples were grouped and tested by geographical region widespread significant genetic structuring was shown in both mtDNA and microsatellite data across the range of *H. guttulatus* (Table 3). The majority of pairwise F_{ST} tests were significant even after sequential Bonferroni correction, the two exceptions being UK v BISCAY and MSP v SPORT. Gene flow estimates calculated in Migrate reveal a complex population structure (Fig. 3) that shares aspects of the pattern revealed in pairwise differentiation tests (Table 3), with high values within UK-BISCAY and SPORT-MSP but much lower values elsewhere. The UK-BISCAY estimates are bidirectional but unequal, with substantially more gene flow southwards, whereas the SPORT-MSP estimates are bidirectional and symmetrical. The Black Sea displays zero gene flow between it and all other regions. However the EMED populations do have genetic exchange with populations from WMED, MSP and SPORT. The STRUCTURE analysis with both admixture and non-admixture models indicated highest support for three genetic clusters among the sampled locations, which are UK-BISCAY, SPORT-MSP-WMED-EMED and BLACK (Fig. 4). Subsequent analysis of just the SPORT-MSP-WMED-EMED cluster shows clear support for divergent clustering of SPORT-MSP and WMED-EMED (Fig. 4) resulting in four overall clusters. As a

precautionary analysis to comply with the conservation management aims of the study, four metapopulations were chosen for demographic coalescent model analysis (UK+BISCAY, SPORT+MSP, WMED+EMED, and BLACK). Henceforth these metapopulations are referred to as N. ATLANTIC (comprising PUK, BFR, CFR, RFR, SSP samples), SW. IBERIA (TPO, PPO, RPO, MSP), MED (ASP, SFR, NITRIT, KGR, CGR) and BLACK (VBU).

Mantel tests to assess correlation of genetic and geographic distance gave a positive and significant relationship among all Atlantic and Mediterranean samples (mtDNA: $r=0.6910$, $p<0.01$; microsatellites: $r=0.5267$, $p<0.05$). Subdivision of the sample sets indicated that the significant relationship was maintained across the samples from the Atlantic Ocean to Malaga site (MSP) ($r=0.5352$ $p<0.001$), but that no correlation existed across the Mediterranean samples ($r=0.3033$, $p>0.05$).

Historical processes

The DIYABC coalescent analysis indicated large values for contemporary effective population size in all four regional metapopulations (N_E of $\sim 730K$ to $1130K$ – Table 4). Estimates of divergence times between the four populations were all relatively recent, ranging from 18Kya between N.ATLANTIC and SW.IBERIA up to 66 Kya between SW.IBERIA and MED (Table 4). Estimates of time since population expansion are even more recent, ranging from 2.4 Kya to 9.5 Kya (Table 4). However, because our models do not include divergence with gene flow, divergence times should be considered as approximations, allowing for the possibility of lineage divergence with gene flow taking place over a longer period of time.

Discussion

Genetic variability

Levels of genetic diversity within species are important to consideration of conservation and management plans, where maintenance of genetic diversity is recommended (Kenchington et al. 2003). The high haplotype number combined with low nucleotide diversity observed in *H. guttulatus* is indicative of recent population expansion (Grant and Bowen 1998) across the species range. There are two exceptions to this range-wide pattern: the most northern population and a population located in the Thau lagoon, a water mass with extremely limited water flow to the Mediterranean Sea, have lower haplotype diversity. Lower diversity in the UK can be explain by Hewitt's (2000) model of colonisation of geographically peripheral range edge sites, whereas the Thau lagoon population is more likely to be a result of inbreeding in a small isolated population (Frankham 2005). Such patterns are common in marine species (Astolfi et al. 2005; Gysels et al. 2004; Teske et al. 2003) both at the extreme limits of the species' range and in isolated sites. Such differences in diversity, however, were

not observed in *H. hippocampus* (Woodall et al. 2011b), which may result from differences in habitat preference between these two seahorse species, with *H. hippocampus* more often found along open coasts whereas *H. guttulatus* is more frequently found in discontinuous habitats such as estuaries and lagoons (Woodall 2009).

The range-wide ubiquity of a common mtDNA haplotype combined with many closely related haplotypes, and regional population groups with differing proportions of private haplotypes, in *H. guttulatus* is congruent with the distribution of microsatellite genotype variation, and a pattern common to other seahorses and is thought to reflect post-bottleneck expansions from a single refugium with ongoing contemporary gene flow (Saarman et al. 2010; Teske et al. 2003; Woodall et al. 2011b). Other syngnathid species, however display different distributions of genetic diversity, so species-specific characteristics need to be discerned and taken into account in management. The Mediterranean lagoon-dwelling pipefish *Syngnathus abaster* has a more complex haplotype network but no shared haplotypes between populations, and similar nucleotide diversity to seahorses (Sanna et al. 2013), suggesting that the fragmented habitat and life history characteristics of the species have resulted in population isolation and breakdown of gene flow following the initial post-glacial expansion. On a larger geographical scale the seahorse *H. erectus* also demonstrates regionality and genomic divergence, with little connectivity between northern and southern populations occupying waters with very different environmental conditions (Boehm et al. 2015).

Population structuring

Genetic analysis revealed a complex pattern of subpopulations and connectivity with the initial regional assignments (Table 1), with four geographically defined lineages indicated: UK to northern Spain; Portugal to Malaga on the Mediterranean coast of Spain; the rest of the Mediterranean; and the Black Sea. There was some evidence for divergence between western and eastern regions of the Mediterranean Sea, but this was to an extent much less than the other divisions and not supported by all analyses (see below). Subpopulation genetic divergence revealed in *H. guttulatus* appears to partially reflect that found in the congeneric and co-distributed short snouted seahorse *H. hippocampus* (Woodall et al. 2011b). It may be expected that both species would have a similar pattern of population differentiation as they can co-occur and have very similar life-history characters. However they show differences in micro-habitat preference (Curtis et al. 2007) and macro-habitat distribution (Woodall 2009). *Hippocampus hippocampus* also has a greater southern latitudinal range and is thought to have undergone more recent range expansion than *H. guttulatus* (Boehm et al. 2013; Teske et al. 2007). This apparent greater structuring of *H. guttulatus* suggests that a different combination of historical and contemporary processes may have contributed to these species' population structure.

Impact of life-history on genetic diversity

In contrast to expectations of very limited dispersal and gene flow predicted from species biology and life history, the pattern of genetic similarity observed within and among geographical regions across the species range suggests that *H. guttulatus* dispersal, although limited in places, is sufficient to maintain long-term gene flow across relatively large distances. The apparent isolation of the Black Sea population, signified by significant inbreeding and genetic differentiation, and breakdown of gene flow across several regions (NW Iberia and Mediterranean coast of southern Spain) illustrates the potential for this species to form segregated populations. The overall genetic similarity across large areas suggests that unsampled stepping-stone populations could be the conduit for genetic exchange between sampled populations, as supported by isolation-by-distance effects across large parts of the range (Palumbi 2003).

Contemporary barriers to gene flow in *H. guttulatus*

Cape Finisterre. A major barrier to gene flow between the northern Spanish and southeast Portuguese sites was supported in *H. hippocampus* (Woodall et al. 2011b), and a similar pattern is consistent with our analysis of *H. guttulatus*. Other studies have observed Cape Finisterre in northwest Spain as being associated with genetic differentiation of marine populations (Neiva et al. 2012; Piñeira et al. 2008; Quesada et al. 1998), although a small-scale study of *H. guttulatus* across this area did not find population differentiation to either side of the cape (Lopez et al. 2015). A more southerly barrier to gene flow, between Rio Mondego and Rio Sado in central Portugal, has been suggested for other marine species (Diekmann et al. 2005; Pascoal et al. 2009). Further small-scale research will be required to elucidate exactly where gene flow breaks between *H. guttulatus* population around the northwestern Iberian Peninsula occur. However the interaction of current and upwelling systems along with fragmented habitat are likely to define the location of the barrier, which could be understood by biophysical oceanographic modelling (Nolasco et al. 2013).

Gibraltar Straits or Almeria/Oran front? Genetic data indicate that the population MSP, in the Alboran Sea, east of the Straits of Gibraltar but west of the Almeria-Oran front (AOF), is part of the SW IBERIA metapopulation (Atlantic coasts). Thus the AOF correlates with *H. guttulatus* population structure and is the likely barrier to genetic exchange between Atlantic Ocean and Mediterranean Sea populations. The Atlantic–Mediterranean biogeographic boundary has been analysed in over 70 studies of many different marine organisms, and both fish (Charrier et al. 2006; Domingues et al. 2007) and invertebrates (Baus et al. 2005; Perez-Losada et al. 2002) show genetic differentiation correlating with the AOF. A number of studies, reviewed in Patarnello et al. (2007), suggest that the AOF is a significant physical barrier to individual dispersal and gene flow.

The Siculo-Tunisian strait. Although to a much lesser extent than other gene flow boundaries identified in the present study, there is some evidence for genetic differentiation between populations of the western and eastern basins of the Mediterranean. The shallow sill of the Siculo-Tunisian straits disrupts local hydrodynamics and current flows, and so hinders genetic exchange between the two basins in a number of marine species (Merjri et al. 2009; Serra et al. 2009) and is thought to be a biogeographic boundary (Bianchi and Morri 2000). The absence of isolation-by-distance effects in *H. guttulatus* across the Mediterranean suggests that the differentiation across the Siculo-Tunisian Strait is worthy of further investigation and decision as to its importance to management of this species.

The Bosphorus Straits and the Black Sea. The Black Sea is geographically isolated with only a narrow connection to the Mediterranean Sea through the Bosphorus Straits. The historic isolation of the Black Sea and its distinct present environmental parameters (Sorokin 2002) suggest that the observed seahorse population structure could be a result of both historic and contemporary conditions. Our coalescent analysis suggests that historically the Black Sea population diverged from that in the Mediterranean roughly 50 Kya, just prior to the last glacial maximum (LGM), followed by a more recent population expansion after the LGM. The present low but significant genetic differentiation of the Black Sea *H. guttulatus* population (Table 3) indicates that it has not yet achieved migration-drift equilibrium with the Mediterranean population since the LGM. Genetic differentiation of Black Sea from eastern Mediterranean fish populations has been reported previously (Debes et al. 2008; Durand et al. 2013; and see Patarnello et al. 2007), but by contrast so has genetic homogeneity in other fish species (Magoulas et al. 2006), including the confamilial pipefish (Wilson & Eigenmann Veraguth 2010). It is likely that *H. guttulatus* has experienced episodic colonisation, isolation and gene flow in the Black Sea during multiple glacial cycles, in common with other fish species such as shad (Faria et al. 2012), but at present the Black Sea appears to harbour a distinct subpopulation of this seahorse.

Historic demographic effects on diversity and distribution

The *H. guttulatus* mtDNA haplotype network is consistent with a past demographic process of population expansion following a bottleneck across the species range. Such a demographic signature of population bottleneck plus expansion of is found in many other marine fishes across the same geographic range (e.g. Domingues et al. 2008), including other Syngnathids (Saarman et al. 2010; Wilson & Eigenmann Verguth 2010; Woodall et al. 2011b). The DIYABC analyses suggest that isolation and divergence of populations of *H. guttulatus* across Europe occurred during the last glacial maximum (66-18 Kya), and that population expansions occurred in all sub-populations after the LGM to the present (<10 Kya). Similar demographic signatures of past

glacial periods are commonly seen in European marine species, and in some populations of Syngnathidae (Maggs et al. 2008; Wilson & Eigenmann Veraguth 2010; Woodall et al. 2011b). In common with other temperate marine species, and in accord with Hewitt's (2000) model, the presence of a common haplotype across the range and higher genetic diversity of the more southern populations (SW IBERIA and Mediterranean in the present study) indicates that these areas harboured larger or refugial populations during previous glacial periods of the Pleistocene, and that the more northern populations of Biscay that exhibit the most extreme signals of expansion (Table 1) may have been extirpated and subsequently recolonized (at least during the more extreme glacial maxima before the LGM).

Conservation Conclusions

Our data indicate substantial genetic diversity and connectivity across the European range of *H. guttulatus*, but also the effects of two substantial barriers to gene flow (and consequent genetic differentiation), at Cape Finisterre and the Bosphorus Straits, and further differentiation across the Almeria-Oran front and between the eastern and western Mediterranean. These patterns reveal that both contemporary processes (life-history and oceanographic features) and historic (paleoclimatic) events influence present population structure of *H. guttulatus*. We suggest that following the initial speciation in the Miocene (Teske et al. 2007), contraction of the species range during Pleistocene glacial maxima to at least one southern European refugial population followed by recurrent expansion and re-colonisation from these sites has been mediated by the isolating mechanism of oceanographic features combined with the low dispersal potential of *H. guttulatus*.

Current genetic structuring and diversity suggests four main *H. guttulatus* metapopulations, with potential subdivision of the east and west Mediterranean, which should be recognised as management units (MU) (Palsboll et al. 2006). In future, further details of genetic differentiation across smaller geographic ranges (additional sub-structuring) and of specific genetic barriers could be used to determine if particular priority should be given to specific populations (Volkman et al. 2014). However current data suggest that the MU designation is robust and should be considered as the basis of a management strategy for this species, which would mean combining range-wide coastal habitat conservation and transboundary planning for protected areas.

Connectivity around the coastline is reliant on suitable habitat for *H. guttulatus*, which should be considered carefully in conservation plans. The population structure observed, suggests that the sedentary nature of this fish is most likely partially offset by the dispersal of juveniles as zooplankton, occasional migration events by adults, and/or dispersal by rafting (Luzzatto et al. 2013).

Coastal ecosystems have many wildly varying environmental parameters, suggesting seahorses often experience non-ideal conditions, which in turn may cause demographic fluctuations (Caldwell & Vincent 2012; Curtis & Vincent 2006; Woodall 2009, 2012). These demographic decreases may be the drivers for the observed genetic differentiation. Additionally, reduced genetic diversity as a result of these localised bottleneck events is thought to be an indicator of extinction risk in threatened species (Frankham 2005). Care should therefore be taken not just to conserve *H. guttulatus* metapopulations but also to protect potential habitat. Indeed this ecosystem management approach is now popular (Pérez-Ruzafa et al. 2008), and an identified international fisheries policy goal (Veitch et al. 2012). *Hippocampus guttulatus* is currently listed as Data Deficient on the IUCN Red List (Woodall 2012) with a suggestion that more information on population demographic changes is required before it can be categorized. Therefore long-term monitoring of known populations is required to determine population trends. In addition, further genetic studies are required, focusing on population connectivity along the coast at the 50-100 Km scale, to determine possible stepping-stone populations and to establish if contemporary gene flow within metapopulations is deemed large enough to ensure long term survival. There is no known targeted fishery for this species, but seahorses are threatened by anthropogenic activities in coastal ecosystems, such as habitat disturbance from aggregate dredging, coastal development, pollution and fishing activity (Vincent et al. 2011). As reported for *H. hippocampus* (Woodall et al. 2011b) there are no Europe-wide conservation measures in place for seahorses, but it is important for management agencies to work internationally due to the transboundary nature of the *H. guttulatus*' range and proposed MUs. In addition, seahorses are globally considered a charismatic flagship species, and because they share habitat with numerous taxa the protection of their populations and habitat can extend to whole ecosystems being protected from harmful activities.

The signatures of the complex history of climate shifts are evident in *H. guttulatus* population structure. This suggests that this species has previously coped with environmental conditions that have caused localised population extinctions. Many extant populations are seen to inhabit regions with large temperature fluctuations (Woodall 2009). However, contemporary climate change will result in changes to the population structure through habitat and hydrodynamic changes, and thus to the location and possibly the composition of the MUs suggested here. Therefore the implications of climate change on *H. guttulatus* would have to be carefully considered and add further justification to the importance of monitoring populations of this fish. In summary, the design of any proposed international management strategies should be informed by the meta-populations

elucidated in this study, but further monitoring of population structure and demography is recommended to ensure the long-term viability of European seahorse populations.

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References

- Arnaud-Haond S, Migliaccio M, Diaz-Almela E, Teixeira S, Van de Vilet MS, Alberto F, Procaccini G, Duarte CM, Serrao EA (2007) Vicariance patterns in the Mediterranean Sea: east-west cleavage and low dispersal in the endemic seagrass *Posidonia oceanica*. *J of Biogeo* 34:963-976
- Astolfi L, Dupanloup I, Rossi R, Bisol PM, Faure E, Congiu L (2005) Mitochondrial variability of sand smelt *Atherina boyeri* populations from north Mediterranean coastal lagoons. *Mar Ecol Prog Ser* 297:233-243
- Bargelloni L, Alarcon JA, Alvarez MC, Penzo E, Magoulas A, Palma J, Patarnello T (2005) The Atlantic–Mediterranean transition: Discordant genetic patterns in two seabream species *Diplodus puntazzo* (Cetti) and *Diplodus sargus* (L.). *Mol Phylogenet Evol* 36:523-535
- Baus E, Darrock DJ, Bruford MW (2005) Gene-flow patterns in Atlantic and Mediterranean populations of the Lusitanian sea star *Asterina gibbosa*. *Mol Ecol* 14:3373-3382
- Beaumont MA, Zhang W, Balding DJ (2002) Approximate Bayesian computation in population genetics. *Genetics* 162:2025-2035
- Beerli P, Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. *Proc Natl Acad Sci USA* 98:4563-4568
- Bianchi NC, Morri C, (2000) Marine biodiversity of the Mediterranean Sea: Situation, problems and prospects for future research. *Mar Poll Bullet* 40:367–376
- Blake RW (1976) On seahorse locomotion. *J Mar Biol Assoc UK* 56:939-949

454 Boehm JT, Waldman J, Robinson JD, Hickerson MJ (2015) Population genomics reveals seahorses
 455 *Hippocampus erectus* of the western mid-Atlantic coast to be residents rather than vagrants. Plos One 10:
 456 e0116219

457 Boehm JT, Woodall LC, Teske PR, Lourie SA, Baldwin C, Waldman J, Hickerman M (2013) Marine dispersal
 458 and barriers drive Atlantic seahorse diversification. J Biogeogr 40:1839-1849

459 Boisseau J (1967) Les régulations hormonales de l'incubation chez un vertèbre male: recherches sur la
 460 reproduction de l'hippocampe. PhD thesis, L'Université de Bordeaux, France

461 Caldwell IR, Vincent ACJ (2013) A sedentary fish on the move: effects of displacement on long-snouted
 462 seahorse (*Hippocampus guttulatus* Cuvier) movement and habitat use. Environ Biol Fish 96:67-75

463 Casado-Amezua P, Goffredo S, Templado J, Machordom A (2012) Genetic assessment of population structure
 464 and connectivity in the threatened Mediterranean coral *Astroides calycularis* (Scleractinia, Dendrophylliidae)
 465 at different spatial scales. Mol Ecol 21:3671-3685

466 Casey SP, Hall HJ, Stanley HF, Vincent ACJ (2004) The origin and evolution of seahorses (genus
 467 *Hippocampus*): a phylogenetic study using the cytochrome b gene of mitochondrial DNA. Mol Phylogenet
 468 Evol 30:261-272

469 Chapuis M-P, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. Mol Biol
 470 Evol 24:621-631

471 Charrier G, Chenel T, Durand JD, Girand M, Quiniou L, Laroche J (2006) Discrepancies in phylogeographical
 472 patterns of two European anglerfishes (*Lophius budegassa* and *Lophius piscatorius*). Mol Phylogenet Evol
 473 38:742-754

474 Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol
 475 9:1657-1659

476 Cornuet J-M, Ravigne V, Estoup A (2010) Inference on population history and model checking using DNA
 477 sequence and microsatellite data with the software DIYABC (v1.0). BMC Bioinformatics 11:401

478 Cornuet J-M, Santos F, Beaumont MA, Robert CP, Marin J-M, Balding DJ, Guillemaud T, Estoup A (2008)
 479 Inferring population history with DIY ABC: a userfriendly approach to approximate Bayesian computation.
 480 Bioinformatics 24:2713-2719

481 Curtis JMR, Ribeiro J, Erzini K, Vincent ACJ (2007) A conservation trade-off? Interspecific differences in
 482 seahorse responses to experimental changes in fishing effort. Aquat Conserv 17:468-484

483 Curtis JMR, Vincent ACJ (2005) Distribution of sympatric seahorse species along a gradient of habitat
484 complexity in a seagrass-dominated community. *Mar Ecol Prog Ser* 291:81-91

485 Curtis JMR, Vincent ACJ (2006) Life history of an unusual marine fish: survival, growth and movement
486 patterns of *Hippocampus guttulatus* Cuvier 1829. *J Fish Biol* 68:707-733

487 Debes PV, Zachos FE, Hanel R (2008) Mitochondrial phylogeography of the European sprat (*Sprattus sprattus*
488 L., Clupeidae) reveals isolated climatically vulnerable populations in the Mediterranean Sea and range
489 expansion in the northeast Atlantic. *Mol Ecol* 17:3873-3888

490 Diekmann OE, Coyer JA, Ferreira J, Olsen JL, Stam WT, Pearson GA, Serrao EA (2005) Population genetics of
491 *Zostera noltii* along the west Iberian coast: consequences of small population size, habitat discontinuity and
492 near-shore currents. *Mar Ecol Prog Ser* 290:89-96

493 Domingues VS, Faria C, Stefanni S, Santos RS, Brito A, Almada VC (2007) Genetic divergence in the Atlantic-
494 Mediterranean Montagu's blenny, *Coryphoblennius galerita* (Linnaeus 1758) revealed by molecular and
495 morphological characters. *Mol Ecol* 16:3592-3605

496 Durand JD, Blel H, Shen KN, Koutrakis ET, Guinand B (2013) Population genetic structure of *Mugil cephalus*
497 in the Mediterranean and Black Seas: a single mitochondrial clade and many nuclear barriers. *Mar Ecol Prog*
498 *Ser* 474:243-261

499 Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing
500 STRUCTURE output and implementing the Evanno method. *Conserv Genet Resour* 4:359-361

501 Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software
502 STRUCTURE: a simulation study. *Mol Ecol* 14:2611-2620

503 Excoffier L, Lisher HEL (2010) Arlequin ver. 3.5: A new series of programs to perform population genetics
504 analysis under Linux and Windows. *Mol Ecol Resour* 10:564-567

505 Faria R, Weiss S, Alexandrino P (2012) Comparative phylogeography and demographic history of European
506 shads (*Alosa alosa* and *A. fallax*) inferred from mitochondrial DNA. *BMC Evol Biol* 12:194

507 Foster SJ, Vincent ACJ (2004) Life history and ecology of seahorses: implications for conservation and
508 management. *J Fish Biol* 65:1-61

509 Frankham R (2005) Genetics and extinction. *Biol Conserv* 126:131-140

510 Galarza JA, Carreras-Carbonell J, Macpherson E, Pascual M, Roques S, Turner GF, Rico C (2009) The
511 influence of oceanographic fronts and early life-history traits on connectivity among littoral fish species.
512 *Proc Natl Acad Sci USA* 106:1473-1478

513 Galbusera PHA, Gillemot S, Jouk P, Teske PR, Hellemans B, Volckaert AMJ (2007) Isolation of microsatellite
514 markers for the endangered Knysna seahorse *Hippocampus capensis* and their use in the detection of a
515 genetic bottleneck. Mol Ecol Notes 7: 638-640

516 Galindo HM, Pfeiffer-Herbert AS, McManus MA, Chao Y, Chai F, Palumbi SR (2010) Seascape genetics along
517 a steep cline: using genetic patterns to test predictions of marine larval dispersal. Mol Ecol 19: 3692-3707

518 Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3.2),
519 Universite de Lausanne, Dorigny, Switzerland

520 Grant WS, Bowen BW (1998) Shallow population histories in deep evolutionary lineages of marine fishes:
521 insights from sardines and anchovies and lessons for conservation J Hered 89: 415-426

522 Green EP, Short FT (2003) World Atlas of Seagrasses. Berkeley, USA, University of California Press

523 Guarniero I, Franzellitti S, Ungaro N, Tommasini S, Piccinetti C, Tinti F (2002) Control region haplotype
524 variation in the central Mediterranean common sole indicates geographical isolation and population
525 structuring in Italian stocks, J Fish Biol 60: 1459-1474

526 Gysels E S, Hellemans B, Patarnello T, Volckaert FAM, (2004) Current and historical gene flow of the sand
527 goby *Pomatoschistus minutus* on the European Continental Shelf and in the Mediterranean Sea, Biol J Linn
528 Soc 83: 561-576

529 Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows
530 95/98/NT. Nucleic Acids Symp Ser 41:95-98F

531 Hewitt G (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907-913

532 Hoarau G, Rijnsdorp AD, Van der Veer HW, Stam WT, Olsen JL (2002), Population structure of plaice
533 (*Pleuronectes platessa* L.) in northern Europe: microsatellite revealed large-scale spatial and temporary
534 homogeneity. Mol Ecol 11:1165-1176

535 Hudson RR, Slatkin M, Maddison WP (1992) Estimation of gene flow from DNA sequence data. Genetics
536 132:583-589

537 Jensen JL, Bohonah AJ, Kelley ST (2005) Isolation by distance, web service. BMC Genet 6:13

538 Jorgensen HBH, Hansen MM, Bekkevold D, Ruzzante DE, Loeschcke V (2005) Marine landscapes and
539 population genetic structure of herring (*Clupea harengus* L.) in the Baltic Sea. Mol Ecol 14:3219-3234

540 Kenchington E, Heino M, Nielsen EE (2003) Managing marine genetic diversity: time for action? ICES J Mar
541 Sci 60:1172-1176

542 Lopez A, Vera M, Planas M, Bouza C (2015) Conservation genetics of threatened *Hippocampus guttulatus* in
 543 vulnerable habitats in NW Spain: temporal and spatial stability of wild populations with flexible polygamous
 544 mating system in captivity. Plos One 10:e0117538
 545 Lourie SA, Foster SJ, Cooper EWT, Vincent ACJ (2004) A guide to the identification of seahorses, Washington
 546 D.C. USA
 547 Lourie SA, Green DM, Vincent ACJ (2005) Dispersal, habitat differences, and comparative phylogeography of
 548 Southeast Asian seahorses (Syngnathidae: *Hippocampus*). Mol Ecol 14:1073-1094
 549 Luzzatto DC, Estalles ML, Diaz de Astarloa JM (2013) Rafting seahorses: the presence of the seahorse
 550 *Hippocampus patagonicus* in floating debris. J Fish Biol 83:677-681
 551 Maggs CA, Castilho R, Foltz D, Henzler C, Jolly MT, Kelly J, Olsen J, Perez KE, Stam W, Vainola R, Viard F,
 552 Wares J (2008) Evaluating valuating signatures of glacial refugia for North Atlantic benthic marine taxa.
 553 Ecology 89:S108-S122
 554 Magoulas A, Castilho R, Caetano S, Marcato S, Patarnello T (2006) Mitochondrial DNA reveals a mosaic
 555 pattern of phylogeographical structure in Atlantic and Mediterranean populations of anchovy (*Engraulis*
 556 *encrasicolus*). Mol Phylogenet Evol 39:734-746
 557 Merji, R, Lo Butto S, Ben Hassine OK, Arculeo M (2009) A study on *Pomatoschistus tortonesei* Miller 1968
 558 (Perciformes, Gobiidae) reveals the Siculo-Tunisian Strait (STS) as a breakpoint for gene flow in the
 559 Mediterranean basin. Mol Phylogenet Evol 53:596-601
 560 Merjri R, Arculeo M, Ben Hassine, OK, Brutto SL (2011) Genetic architecture of the marbled goby
 561 *Pomatoschistus marmoratus* (Perciformes, Gobiidae) in the Mediterranean Sea. Mol Phylogenet Evol
 562 58:395-403
 563 Milano I, Babbucci M, Cariani A, Atanassova M, Bekkevold D, Carvalho GR, Espineira M, Fiorentino F,
 564 Garofalo G, Geffen AJ, Hansen JH, Helyar SJ, Nielsen EE, Ogden R, Patarnello T, Stagoni M, Consortium
 565 F, Tinti F, Bargelloni L (2014) Outlier SNP markers reveal fine-scale genetic structuring across European
 566 hake populations (*Merluccius merluccius*). Mol Ecol 23:118-135
 567 Neiva J, Pearson GA, Valero M, Serrao EA (2012) Fine-scale genetic breaks driven by historical range dynamics
 568 and ongoing density-barrier effects in the estuarine seaweed *Fucus ceranoides* L. BMC Evol Biol 12:78
 569 Nicastro KR, Zardi GI, McQuaid CD, Teske PR, Barker NP (2008) Coastal topography drives genetic structure
 570 in marine mussels. Mar Ecol Prog Ser 368:189-195

571 Nolasco R, Dubert J, Dominges CP, Cordeiro Piers A, Queiroga H (2013) Model-derived connectivity patterns
 572 along the western Iberian Peninsula: asymmetrical larval flow and source-sink cell. *Mar Ecol Prog Ser*
 573 485:123-142
 574 Palsboll PJ, Berube M, Allendorf FW (2006) Identification of management units using population genetic data.
 575 *Trends Ecol Evol* 22:11-16
 576 Palumbi SR (2003) Population genetics, demographic connectivity and the design of marine reserves. *Ecol Appl*
 577 13:S146-S158
 578 Pardo BG, López A, Martínez P, Bouza C (2006) Novel microsatellite loci in the threatened European long-
 579 snouted seahorse (*Hippocampus guttulatus*) for genetic diversity and parentage analysis. *Conserv Genet*
 580 8:1243-1245
 581 Pascoal S, Creer S, Taylor MI, Queiroga H, Carvalho G, Mendo S (2009) Development and application of
 582 microsatellites in *Carcinus maenas*: genetic differentiation between northern and central Portuguese
 583 populations. *Plos One* 4: e7268.
 584 Patarnello T, Volckaert FA, Castilho R (2007) Pillars of Hercules: is the Atlantic-Mediterranean transition a
 585 phylogeographical break? *Mol Ecol* 16:4426-4444
 586 Perante NC, Pajaro MG, Meeuwig JJ, Vincent ACJ (2002) Biology of a seahorse species, *Hippocampus comes*
 587 in the central Philippines. *J Fish Biol* 60:821-837
 588 Perez-Losada M, Guerra A, Carvalho GR, Sanjuan A, Shaw PW (2002) Extensive population subdivision of the
 589 cuttlefish *Sepia officinalis* (Mollusca: Cephalopoda) around the Iberian Peninsula indicated by microsatellite
 590 DNA variation. *Heredity* 89:417-424
 591 Pérez-Ruzafa A, Marcos C, García-Charton JA, Salas F (2008) European marine protected areas (MPAs) as
 592 tools for fisheries management and conservation. *J Nat Conserv* 16:187-192
 593 Piñeira J, Quesada H, Rolán-Alvarez E, Caballero A (2008) Genetic discontinuity associated with an
 594 environmentally induced barrier to gene exchange in the marine snail *Littorina saxatilis*. *Mar Ecol Prog Ser*
 595 357:175-184
 596 Planes S, Jones GP, Thorrold SR (2009) Larval dispersal connects fish populations in a network of marine
 597 protected areas. *Proc Natl Acad Sci USA* 106:5693-5697
 598 Pritchard J, Stephens M, Donnelly P (2000) Inference of population structure from multilocus genotype data.
 599 *Genetics* 155:945-959

600 Quesada H, Gallagher C, Skibinski DAG, Skibinski DOF (1998) Patterns of polymorphism and gene flow of
 601 gender- associated mitochondrial DNA lineages in European mussel populations. *Mol Ecol* 7:1041-1051
 602 Quinteiro J, Rodriguez-Castro J, Rey-Mendez M (2007) Population genetic structure of the stalked barnacle
 603 *Pollicipes pollicipes* (Gmelin, 1789) in the North Eastern Atlantic: influence of coastal currents and
 604 mesoscale hydrographic structures. *Mar Biol* 153:47-60
 605 Ryman N, Palm S (2006) POWSIM: A computer program for assessing statistical power when testing for genetic
 606 differentiation. *Mol Ecol* 6:600-602
 607 Riccioni G, Landi M, Ferrara, G, Milano I, Cariani A, Zane L, Sella M, Barbujan G, Tinti F (2010) Spatio-
 608 temporal population structuring and genetic diversity retention in depleted Atlantic Bluefin tuna of the
 609 Mediterranean Sea. *Proc Natl Acad Sci USA* 107:2102-2107
 610 Rousset F (2008) GENEPOP'007: a complete re-implementation of the genepop software for Windows and
 611 Linux. *Mol Ecol Notes* 8:103-106
 612 Saarman NP, Louie KD, Hamilton H (2010) Genetic differentiation across eastern Pacific oceanographic
 613 barriers in the threatened seahorse *Hippocampus ingens*. *Conserv Genet* 11:1989-2000
 614 Sanna D, Biagi F, Alaya HB, Maltagliati F, Addis A, Romero A, De Juan J, Quignard J-P, Castelli A, Franzoi P,
 615 Torricelli P, Casu M, Carcupino M, Francalacci P (2013) Mitochondrial DNA variability of the pipefish
 616 *Syngnathus abaster*. *J Fish Biol* 82: 856-876
 617 Schunter C, Carreras-Carbonell J, Macpherson E, Tintore J, Vidal-Vijande E, Pascual A, Guidetti P, Pascual M
 618 (2011) Matching genetics with oceanography: directional gene flow in a Mediterranean fish species. *Mol*
 619 *Ecol* 20:5167-5181
 620 Serra IA, Innocenti AM, Dimaida G, Calvo S, Migliaccio M, Zambianchi E, Pizzigalli C, Arnaud-Haond S,
 621 Duarte CM, Serrao EA, Procaccini G (2010) Genetic structure in the Mediterranean seagrass *Posidonia*
 622 *oceanica*: Disentangling past vicariance events from contemporary patterns of gene flow. *Mol Ecol* 19:557-
 623 568
 624 Sorokin YI (2002) *The Black Sea: Ecology and Oceanography*. Backhuis Publishing, Leiden: Netherlands.
 625 Swofford DL (2003) PAUP*. *Phylogenetic Analysis Using Parsimony (* and other methods)*. Sinauer
 626 Associates, Sunderland, MA, USA
 627 Teske PR, Cherry MI, Matthee CA (2003) Population genetics of the endangered Knysna seahorse,
 628 *Hippocampus capensis*. *Mol Ecol* 12:1703-1715

629 Teske PR, Hamilton H, Matthee CA, Barker NP (2007) Signatures of seaway closures and founder dispersal in
 630 the phylogeny of a circumglobally distributed seahorse lineage. *BMC Evol Biol* 7:138-
 631 Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface:
 632 flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*
 633 25:4876-4882
 634 Vandendriessche S, Messiaen M, Vincx M, Degraer S (2005) Juvenile *Hippocampus guttulatus* from a neuston
 635 tow at the French-Belgian border. *Belg J Zool* 135:101-102
 636 Veitch L, Dulvy NK, Koldewey HJ, Lieberman S, Pauly D, Roberts CM, Rodgers AD, Baillie JEM (2012)
 637 Avoiding Empty Ocean Commitments at Rio+20. *Science* 336:1383-1385
 638 Vincent AC, Foster SJ, Koldewey HJ (2011) Conservation and management of seahorses and other
 639 syngnathidae. *J Fish Biol* 78:1681-1724
 640 Volkmann L, Martyn I, Moulton V, Spillner A, Mooers AO (2014) Prioritizing populations for conservation
 641 using phylogenetic networks. *Plos One* 9:e88945
 642 Ward RD, Woodmark M, Skibinski DOF (1994) A comparison of genetic diversity levels in marine, freshwater,
 643 and anadromous fishes. *J Fish Biol* 44:213-232
 644 Wilson AB (2006) Genetic signature of recent glaciation on populations of a near-shore marine fish species
 645 (*Syngnathus leptorhynchus*). *Mol Ecol* 15:1857-1871
 646 Wilson AB, Eigenmann Veraguth I (2010) The impact of Pleistocene glaciation across the range of a widespread
 647 European coastal species. *Mol Ecol* 19:4535-4553
 648 Winnepeninckx B, Backeljau T, Dewachter R (1993) Extraction of high molecular-weight DNA from molluscs,
 649 *Trends Genet* 9:407
 650 Woodall LC (2009) The population genetics and mating systems of European seahorses. PhD thesis, Royal
 651 Holloway-University of London, UK
 652 Woodall LC (2012) *Hippocampus guttulatus*. In: *IUCN 2013. IUCN Red List of Threatened Species*, Version
 653 2013.1 <http://www.iucnredlist.org>. Assessed 24 November 2013
 654 Woodall LC, Jones R, Zimmerman B, Guillaume S, Stubbington T, Shaw P, Koldewey HJ (2012) Partial fin-
 655 clipping as an effective tool for tissue sampling seahorses, *Hippocampus spp.* *J Mar Biol Assoc UK* 92:1427-
 656 1432
 657 Woodall LC, Koldewey HJ, Shaw PW (2011a) Serial monogamy in the European long-snouted seahorse
 658 *Hippocampus guttulatus*. *Conserv Genet* 12:1645-1649

659 Woodall LC, Koldewey HJ, Shaw PW (2011b) Historical and contemporary population genetic connectivity of
660 the European short-snouted seahorse *Hippocampus hippocampus* and implications for management. J Fish
661 Biol 78:1738-1756

662 Zarraonaindia I, Iriondo M, Albaina A, Pardo MP, Manzano C, Grant WS, Irigoien X, Estonba A (2012)
663 Multiple SNP markers reveal fine-scale population and deep phylogeographic structure in European anchovy
664 (*Engraulis encrasicolus* L.). Plos One 7:e42201

Figure Legends

Figure 1. Map of *Hippocampus guttulatus* sample sites and potential oceanographic barriers along the European coastline including the regional groupings assigned to populations (in brackets). Proposed oceanographic barriers to effective dispersal / gene flow in *H. guttulatus*: 1 Brittany; 2 Cape Finisterre; 3 Gibraltar straits; 4 Almeria-Oran front; 5 Siculo-Tunisian front; 6 Bosphorus straits.

Figure 2. *Hippocampus guttulatus* mtDNA haplotype network based on concatenated partial Control Region and cytochrome b sequences. Haplotypes are shown with size proportional to observed frequency, and segments represent the four proposed regional metapopulations. Lines indicate single mutations and black squares unobserved intermediate haplotypes.

Figure 3. Map of *Hippocampus guttulatus* migration rates estimated using MIGRATE. The thicker the line the larger the migration rate and the dashed line shows no migrate exchange is suggested in any direction.

Figure 4. *Hippocampus guttulatus* population structure inferred by STRUCTURE analysis; for the whole geographic region (a) and for the central regions (SW. IBERIA and MED) (b).

680 Figure 1

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683 Figure 2
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Figure 3

Figure 4

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694 **Tables**

695 **Table 1** *Hippocampus guttulatus*: Sample information- sample location, sample code and biogeographic region.
696 Sample size sequenced (S), number of haplotypes (H), number of private haplotypes (P), haplotype diversity (h)
697 and nucleotide diversity (π). Haplotype and nucleotide diversity are only given when sample size ≥ 15 and for
698 all regions.

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Location	Code	Region	S	H	P	h	π
Poole, UK	PUK	UK	15	4	0	0.73	0.001
Brest, France	BFR	BISCAY	2	2	0		
Le Croisic, France	CFR	BISCAY	3	2	0		
La Rochelle, France	RFR	BISCAY	5	4	1		
Arcachon, France	AFR	BISCAY	26	12	4	0.89	0.002
San Sebastian, Spain	SSP	BISCAY	3	2	1		
		BISCAY	39	15	6	0.89	0.002
Troia, Portugal	TPO	SPORT	24	13	3	0.88	0.002
Portimao, Portugal	PPO	SPORT	26	15	5	0.90	0.003
Ria Formosa, Portugal	RPO	SPORT	29	19	6	0.95	0.003
		SPORT	79	30	15	0.91	0.003
Malaga, Spain	MSP	MSP	19	12	1	0.94	0.003
Alicante, Spain	ASP	WMED	4	3	1		
Sete, France	SFR	WMED	26	7	4	0.66	0.001
Napoli, Italy	NIT	WMED	1	1	0		
		WMED	31	9	5	0.66	0.001
Riccione, Italy	RIT	EMED	2	2	0		
Kalamaki, Greece	KGR	EMED	14	9	4	0.88	0.002
Chalkida, Greece	CGR	EMED	13	9	5	0.92	0.002
		EMED	29	17	9	0.91	0.002
Varna, Bulgaria	VBU	BLACK	24	16	13	0.91	0.003

Table 2 *Hippocampus guttulatus* summary statistics for genetic variation across five microsatellite loci and the nine samples where n>14. Sample size (n), haplotype diversity (h), number of alleles (N_a), expected and observed heterozygosity (H_E and H_O), F_{IS} = inbreeding coefficient. Significance *=p<0.05 and **=p<0.01. All regions are represented by a single site, apart from SPORT (denoted by ^a).

Locus	Populations								
	PUK	AFR	TPO ^a	PPO ^a	RPO ^a	MSP	SFR	GRE	VBU
n	15	41	36	42	50	19	24	27	59
h	0.44	0.45	0.38	0.37	0.39	0.37	0.35	0.44	0.34
Hgu4									
N _a	3	4	4	5	8	4	5	2	5
H _O	0.13	0.24	0.25	0.43	0.34	0.37	0.38	0.30	0.22
H _E	0.13	0.23	0.22	0.36	0.36	0.37	0.51	0.26	0.25
F _{IS}	-0.02	-0.09	-0.13	-0.16	-0.11	-0.01	0.27*	-0.16	0.11*
Hgu12									
N _a	4	3	3	3	3	3	2	3	1
H _O	0.20	0.10	0.17	0.21	0.26	0.21	0.21	0.15	0.00
H _E	0.19	0.09	0.16	0.21	0.24	0.20	0.19	0.14	0.00
F _{IS}	-0.04	-0.03	-0.05	0.00	0.02	-0.06	-0.10	-0.04	NA
Hcaμ11									
N _a	8	14	13	15	15	9	7	11	11
H _O	1.00	0.80	0.69	0.88	0.82	0.74	0.62	0.67	0.63
H _E	0.80	0.81	0.74	0.81	0.87	0.84	0.54	0.74	0.72
F _{IS}	-0.27	0.00	0.06	-0.08	-0.02*	0.13**	-0.15	0.10	0.14**
Hcaμ25									
N _a	3	5	5	4	5	4	3	5	3
H _O	0.53	0.54	0.36	0.33	0.24	0.32	0.08	0.15	0.07
H _E	0.52	0.53	0.47	0.35	0.27	0.33	0.08	0.27	0.07
F _{IS}	-0.02	0.01	0.24**	0.05	0.21	0.04	-0.01	0.46*	-0.02
Hcaμ27									
N _a	4	6	6	4	7	3	6	12	10
H _O	0.53	0.71	0.36	0.09	0.18	0.11	0.33	0.74	0.54
H _E	0.56	0.61	0.32	0.09	0.19	0.10	0.43	0.78	0.63
F _{IS}	0.06	-0.18	-0.12	-0.02	0.38	-0.01	0.23	0.06	0.15**
All									
N _a	4.4	6.4	5.8	6.2	7.6	4.6	4.6	6.6	6.0
H _O	0.48	0.48	0.37	0.39	0.36	0.35	0.33	0.4	0.29
H _E	0.44	0.45	0.38	0.36	0.39	0.37	0.35	0.44	0.33
F _{IS}	-0.09	-0.06	0.04	-0.06	0.04	0.06	0.08	0.09	0.13**

706 **Table 3** *Hippocampus guttulatus* genetic differentiation among regional populations (see text and Table 1 for
707 definition) F_{ST} values for mtDNA are below the diagonal and nDNA microsatellites above diagonal, significance
708 levels: ; ** = $p < 0.01$; *** = $p < 0.001$ all remain significant following Bonferroni correction.

709 a)

	UK	BISCAY	SPORT	MSP	WMED	EMED	BLACK
UK		0.012	0.177***	0.182***	0.220***	0.118***	0.155***
BISCAY	0.016		0.103***	0.098***	0.169***	0.094***	0.125***
SPORT	0.119***	0.119***		0.000	0.050***	0.062***	0.065***
MSP	0.196***	0.178***	0.212		0.065***	0.072***	0.079***
WMED	0.202***	0.170***	0.083***	0.158***		0.052***	0.057***
EMED	0.122***	0.098***	0.182***	0.089**	0.064***		0.026***
BLACK	0.124***	0.138***	0.081***	0.112***	0.080***	0.036**	

710 **Table 4** DIYABC estimates of A) contemporary effective population size (Ne) and population expansion, and B) time since divergence for regional populations of

711 *Hippocampus guttulatus*

712 A

Parameters for Regional Populations	Modern Ne (individuals)	Quartiles 2.5-9.75%	Time of size change (years)	Quartiles 2.5-97.5%	Pre size change Ne	Quartiles 2.5-9.75%
BISCAY	736,000	234,000 - 1,450,000	3,730	614-47,500	15,200	6,910-1,270,000
SW.IBERIA	771,000	289,000 - 1,450,000	8,950	522-45,400	269,000	55,100-1,440,000
MED	1,130,000	474,000 - 1,480,000	9,520	1,210-70,300	214,000	54,700 – 1,460,000
BLACK	765,000	218,000 - 1,460,000	2,460	1,050-67,700	170,000	30,400-1,380,000

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714 B

Parameters for Regional Populations	Divergence times (years)	Quartiles 5-95%
T1 S.IBERIA and BISCAY	18,300	7,400-70,400
T2 MED and BLACK	47,100	19,100-87,800
T3 S.IBERIA and MED	66,000	32,300-116,000

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