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

Woodall, L.C.[1,2], Koldewey, H. J.[3] Boehm J.T.[4] & Shaw, P.W.[1,5]

[1]Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK

[2]Natural History Museum, Cromwell Road, London, SW7 5BD, UK

[3]Zoological Society of London, Regent's Park, London, NW1 4RY, UK

[4]City College of New York, New York, NY, 10031, USA

[5]Aberystwyth University, Penglais Aberystwyth, Ceredigion, SY23 3DA, UK

Corresponding Author:

Lucy Woodall

l.woodall@nhm.ac.uk

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

67 divergence of marine species, such as the Straits of Sicily and the Almeria-Oran frontal system in the
68 Mediterranean Sea (Galarza et al. 2009; Patarnello et al. 2007; Schunter et al. 2011).
69 Seahorses have distinct life-history characteristics that have led researchers to hypothesize limited
70 connectivity among patchily distributed populations, as well as making them more vulnerable to habitat
71 destruction and overexploitation (reviewed by Vincent et al. 2011). Predictions of low individual dispersal
72 capacity result from traits such as internal fertilisation and brooding of young, a short planktonic juvenile phase
73 (Boisseau 1967), and adult site fidelity, small home range (Curtis & Vincent 2006) and weak swimming ability
74 (Blake 1976). However seahorses are known to move up to 150m daily, within a lagoon system (Caldwell &
75 Vincent 2013) and have the potential for occasional migration events by rafting (Luzzatto et al. 2013; Perante et
76 al. 2002; Vandendriessche et al. 2005). In addition, most seahorse species are socially (Foster & Vincent 2004)
77 and genetically serially monogamous (Woodall et al. 2011a), which could result in a lower effective population
78 size due to limited parental crossings. Seahorses therefore exhibit characteristics that suggest highly structured
79 genetic populations.
80 *Hippocampus guttulatus*, Cuvier 1829, is distributed along coasts of the North-East Atlantic from the
81 English Channel in the north to NW Africa in the south, and throughout the Mediterranean Sea and Black Sea
82 (Lourie et al. 2004). Like many seahorses, *H. guttulatus* is a shallow coastal and estuarine dweller, often
83 associated with seagrass beds (Curtis & Vincent 2005), potentially limiting dispersal across deep open water.
84 Throughout its range these habitats are disjunct (Green & Short 2003) and as such this species exhibits non-
85 continuous populations, which can reduce the chance of nearby populations mixing. Seahorses such as *H.*
86 *guttulatus* are thus well placed to elucidate the relative influence of life history traits versus environmental and
87 historical climatic factors in determining population connectivity.
88 A single recent study has assessed genetic variation in *H. guttulatus* across a small part of the species'
89 geographic range (NW Iberian Peninsula), and found no significant barriers to gene flow (Lopez et al. 2015),
90 but studies of three other syngnathid species from areas more representative of the geographic range of *H.*
91 *guttulatus* suggest more defined population differentiation in such species. Contemporary population structure
92 of the pipefish *Syngnathus typhle* has been shown to be influenced by Pleistocene glaciations, with post glacial
93 recolonisation effects evident in movement of the geographic range north and eastwards (Wilson & Eigenmann
94 Veraguth 2010), whilst another pipefish (*S. abaster)* displays significant post-glacial fragmentation and
95 differentiation (Sanna et al. 2013). Similarly, both contemporary (i.e. oceanographic barriers) and historic
96 factors (i.e. Pleistocene glaciation) were identified as shaping the population structure across European waters in

97 *Hippocampus hippocampus*, with no evidence for the limited propagule connectivity expected in this family
98 (Woodall et al. 2011b).
99 In this study mtDNA and nuclear DNA (microsatellite) markers were applied to samples from the entire
100 geographic range of *H. guttulatus* to: investigate contemporary genetic population structure; identify potential
101 barriers to gene flow; infer demographic history, including times of population divergence and range expansion;
102 and propose conservation and management practices in the light of data from this and other European seahorse
103 species.
104 **Materials and Methods**
105 Sample acquisition and DNA extraction
106 Specimens were collected from 17 locations across the NE Atlantic Ocean, northern Mediterranean Sea and
107 Black Sea, covering over 6000 km of coastline and with a range of 60-1200 km between neighbouring sites (Fig
108 1, Table 1). Seahorses generally live in low densities, are cryptic and are not commercially targeted in Europe,
109 so they are particularly difficult to sample. As a result at some sites it was necessary to re-sample over
110 successive days and/or consecutive years (site MSP). Tissue samples were collected from each individual *in situ*
111 underwater and non-lethally to minimise impacts on individuals and populations (see Woodall et al. 2012).
112 Genomic DNA was isolated from 3-4 mm^2 of dorsal fin tissue using a standard cetyltrimethyl ammonium
113 bromide (CTAB) chloroform/isoamyl alcohol DNA extraction method (after Winnepenninckx, et al. 1993).
114 Mitochondrial DNA sequencing
115 Fragments of mitochondrial DNA in the hypervariable 5' end of the Control Region (CR) and the
116 cytochrome b gene (cyt*b*) were amplified for a maximum of 29 specimens from each of the 17 range-wide
117 locations (Table 1). The CR was amplified using seahorse-specific primer HCAL2 (Teske et al. 2003) and *H.*
118 *guttulatus*-specific primer HIPPCONR (5'AAG CCG AGC GTT CTC TCC 3'). The cyt*b* was amplified using
119 primer SHORSE 5.3L (Casey et al. 2004) and *H. guttulatus*-specific primer GUTTCYTB-R (5' AGG GGG TTC
120 TAC AGG CAT TAC 3'). Each 50µl PCR reaction contained: 5µl 10X manufacturer provided buffer, 2.5µl
121 $MgCl_2$ (50mM - Bioline, UK), 5µl deoxynucleotide triphosphate mix (dNTP) (1.25nM), 1.2 µl of each primer
122 (10µM), 0.25µl Taq polymerase (5U/µl - Bioline, UK), 14.25µl H_2O and 20µl template DNA (10-50 ng). The
123 PCR profile was composed of an initial denaturation step (2 min at 94 °C), followed by 35 cycles of
124 denaturation (30 s at 94 °C), annealing (30s at 50 °C) and extension (60s at 72 °C), and a final extension step (2
125 min at 72 °C).

126 Amplified products were purified, using either PCR purification kit (Qiagen) or Exonuclease 1-Shrimp
127 Alkaline phosphatase protocols, sequenced in both directions by Macrogen (Korea), then deposited in Genbank
128 (Accession numbers: KM061952 to KM062016).
129 Amplification and screening of microsatellites
130 Twenty-five previously developed seahorse-specific microsatellite primers (Galbusera et al. 2007; Pardo et
131 al. 2006) were tested for amplification, allelic variation, null alleles and stutter bands. Five polymorphic
132 microsatellite loci were selected for final screening (Hgu4, Hgu12, Hcaμ11, Hcaμ25 & Hcaμ27); the other loci
133 either failed to amplify or were monomorphic. In total 313 specimens were genotyped from ten locations for a
134 minimum of 15 individuals per site (Table 2). Loci were amplified separately in 10 μl reactions containing 2 μl
135 template DNA (1–5 ng), 1 μl manufacturer-provided buffer, 0.6 μl $MgCl^2$ (50 mM-Bioline), 1 μl dNTP mix
136 (1.25 mM), 0.25 μl of each primer (10 μM, one being Cy5' labelled), 0.05 μl Taq polymerase (5U/μl-Bioline).
137 The thermocycle profile comprised an initial denaturing step (3 min at 95°C), followed by 35 cycles of
138 denaturing (30 s at 95°C), annealing (30 s at 50°C (Hgu12), 53°C (Hcaμ11 and Hcaμ25) or 55°C (Hgu4 and
139 Hcaμ27), and extension (30 s at 72°C), with a final extension step (3 min at 72°C)). PCR products were run on
140 6% denaturing polyacrylamide gels in an ALFexpressII automated DNA sequencer (Amersham Pharmacia) and
141 allele sizes scored using Fragment Manager v2.9 (Amersham Pharamcia).
142 Genetic Diversity
143 Sequence chromatographs were manually checked for errors and edited unambiguously in BIOEDIT v7.2.5
144 (Hall 1999), then consensus sequences were aligned using CLUSTAL X (Thompson et al. 1997). Genetic
145 diversity indices of haplotype diversity (h) and nucleotide diversity (π) were calculated in ARLEQUIN v3.5.1.3
146 (Excoffier & Lischer 2010). Diversity was calculated for all sample locations and regions with a sample size of
147 15 or larger. Genealogy networks were used to visualise nucleotide sequence divergence and genetic
148 relationships between haplotypes; implemented in TCS v1.21 (Clement et al. 2000).
149 Microsatellite locus number of alleles, conformity with Hardy Weinberg (HW) expectation and linkage
150 disequilibrium were computed in GENEPOP v4.0 (Rousset 2008). Observed and expected heterozygosity were
151 calculated in ARLEQUIN. Due to small samples sizes sites CGR and KGR were pooled to form a single Greek
152 sample (GRE), after testing for allele frequency conformity between the individual samples.
153 Power Analysis
154 No evidence of null alleles was detected within any microsatellite locus using FreeNA (Chapuis & Estoup
155 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 $5x10^5$ and $1x10^6$ 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 v.2.03 (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 (Ne) 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 cyt*b* 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 (π=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} ≥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 of 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 ~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. One 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.

306 Impact of life-history on genetic diversity

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

316 Contemporary barriers to gene flow in *H. guttulatus*

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

328 *Gibraltar Straits or Almeria/Oran front?* Genetic data indicate that the population MSP, in the Alboran Sea, east
329 of the Straits of Gibraltar but west of the Almeria-Oran front (AOF), is part of the SW IBERIA metapopulation
330 (Atlantic coasts). Thus the AOF correlates with *H. guttulatus* population structure and is the likely barrier to
331 genetic exchange between Atlantic Ocean and Mediterranean Sea populations. The Atlantic–Mediterranean
332 biogeographic boundary has been analysed in over 70 studies of many different marine organisms, and both fish
333 (Charrier et al. 2006; Domingues et al. 2007) and invertebrates (Baus et al. 2005; Perez-Losada et al. 2002)
334 show genetic differentiation correlating with the AOF. A number of studies, reviewed in Patarnello et al. (2007),
335 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; Serrra 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 Bosporus Straits and the Black Sea. The Black Sea is geographically isolated with only a narrow connection to the Mediterranean Sea through the Bosporus 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

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

374 **Conservation Conclusions**

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

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

391 Connectivity around the coastline is reliant on suitable habitat for *H. guttulatus,* which should be considered
392 carefully in conservation plans. The population structure observed, suggests that the sedentary nature of this fish
393 is most likely partially offset by the dispersal of juveniles as zooplankton, occasional migration events by adults,
394 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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665 **Figure Legends**

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

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

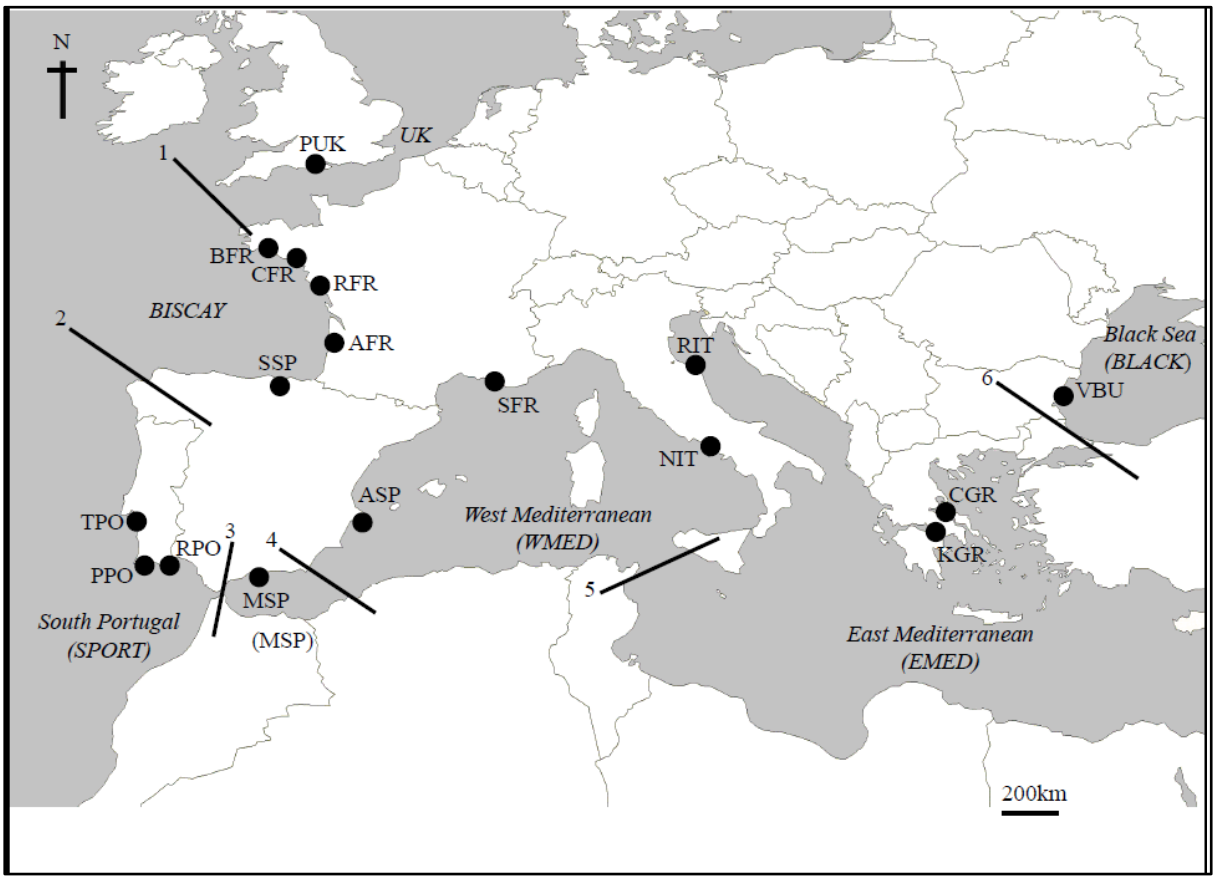
674 **Figure 3.** Map of *Hippocampus guttulatus* migration rates estimated using MIGRATE. The thicker the line the
675 larger the migration rate and the dashed line shows no migrate exchange is suggested in any direction.

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

678

679

680 Figure 1



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683 Figure 2

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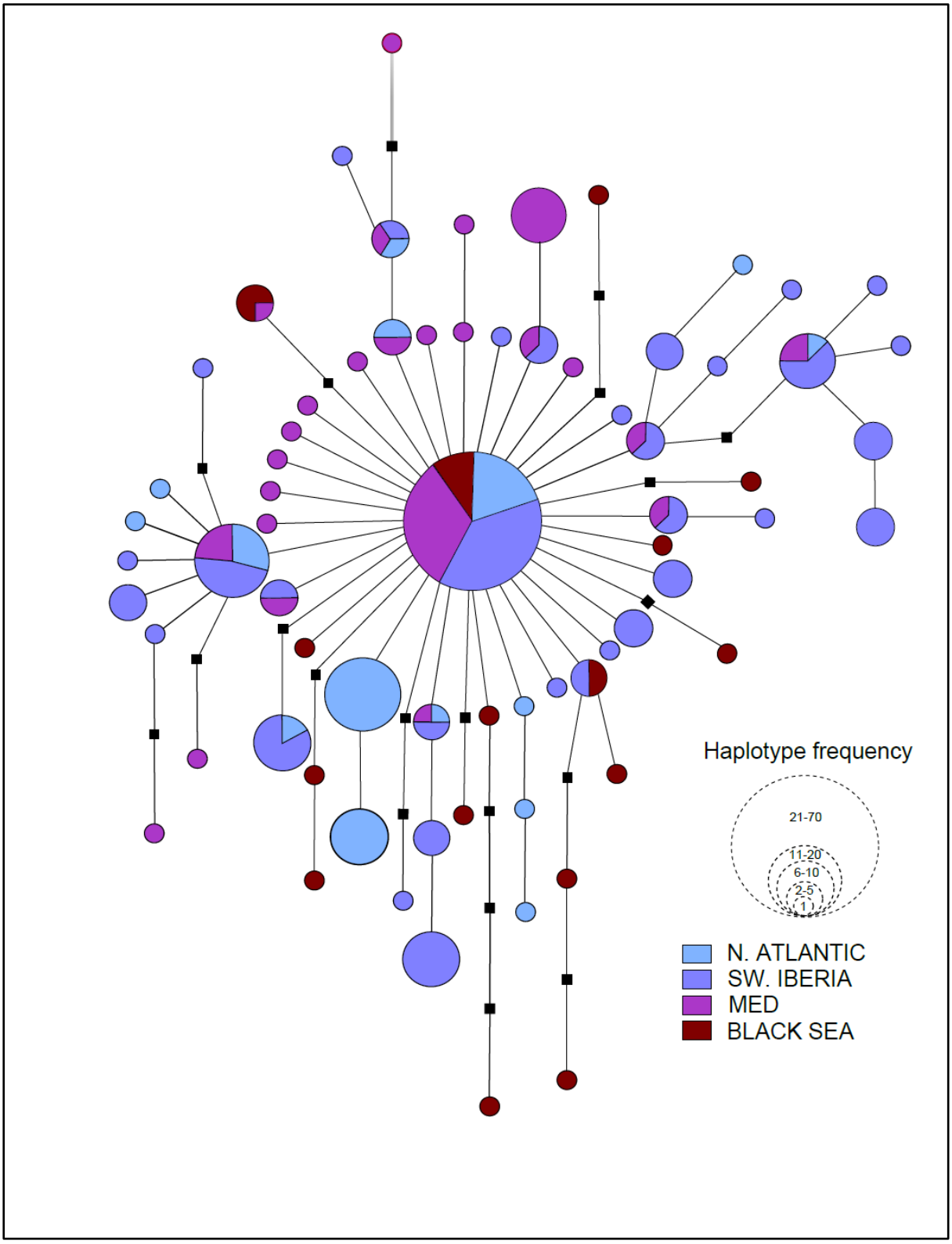

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687 Figure 3

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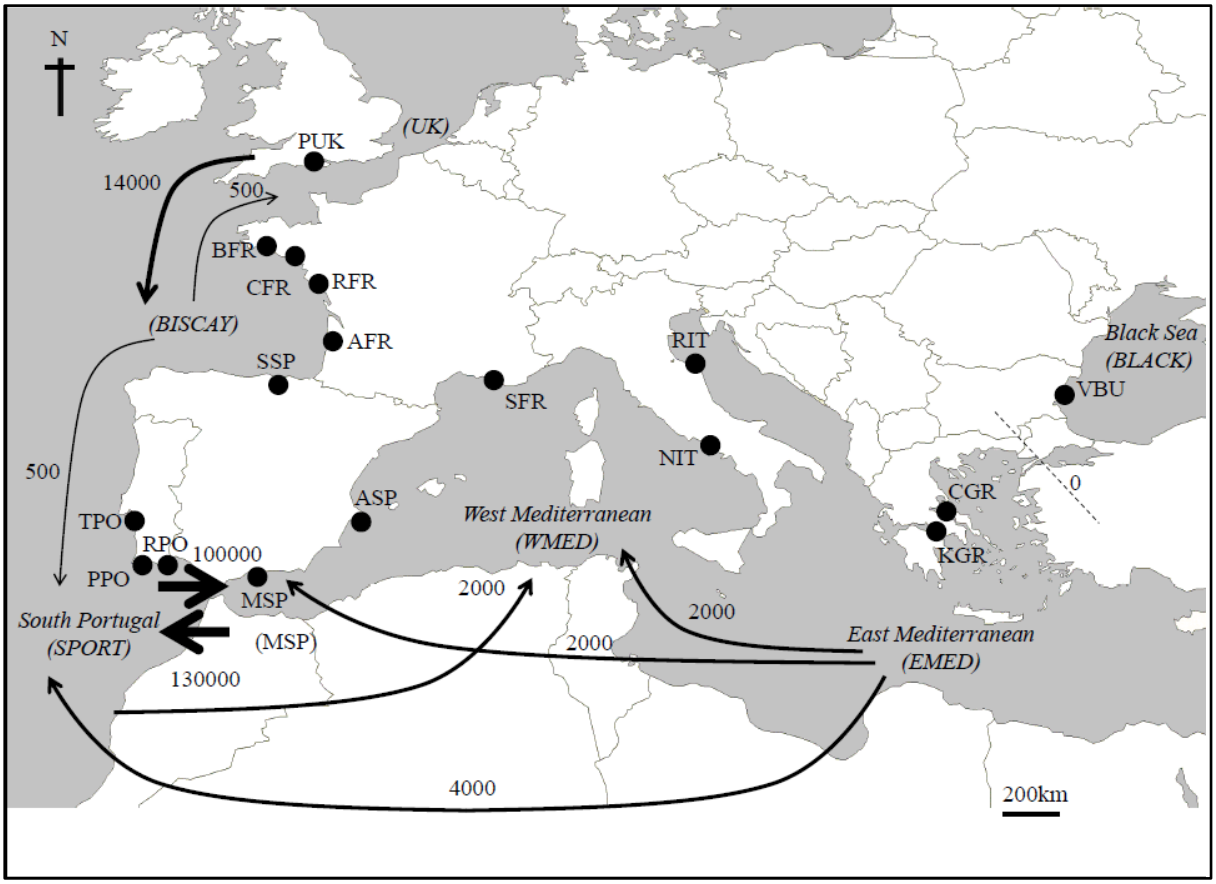

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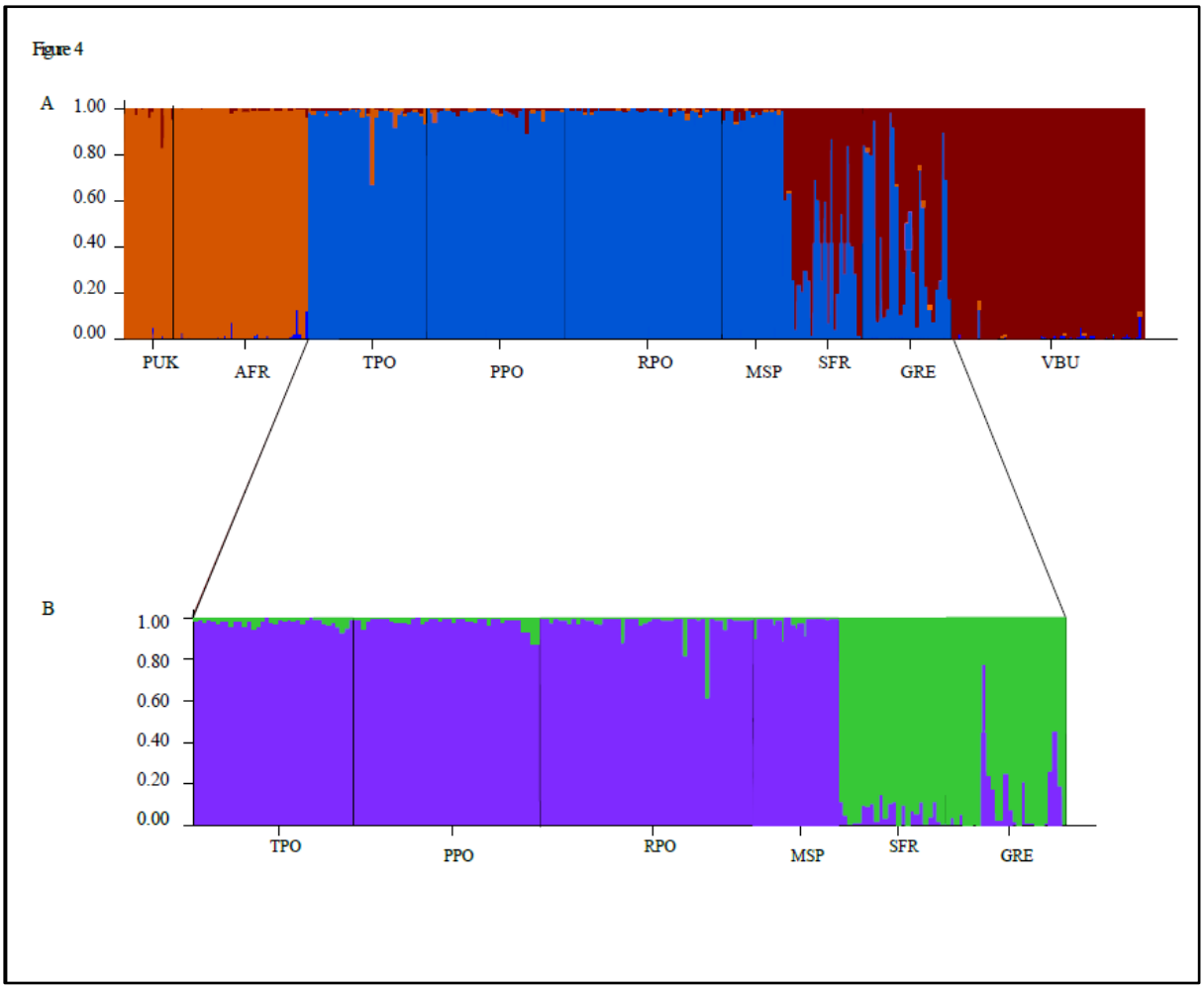
Figure 4
A
1.00
0.80
0.60
0.40
0.20
0.00
PUK
AFR
TPO
PPO
RPO
MSP
SFR
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B
1.00
0.80
0.60
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0.20
0.00
TPO
PPO
RPO
MSP
SFR
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694 **Tables**

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.

699

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

713

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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