*Aquatic Conservation: Marine and Freshwater Ecosystems*

WILEY

ARTICLE OPEN ACCESS

# Genetic Diversity and Connectivity of Syngnathid Fish in Spanish National Parks: Conservation Insights From Protected Marine Ecosystems

Manuel Vera[1] | Belén G. Pardo[1] | Miquel Planas[2] | Inés Castejón-Silvo[3] | Carmen Bouza[1]

[1]Departamento de Zoología, Genética y Antropología Física, Facultad de Veterinaria, Campus Terra, Universidad de Santiago de Compostela, Lugo, Spain | [2]Departamento de Ecología y Recursos Marinos, Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain | [3]Instituto Mediterráneo de Estudios Avanzados (IMEDEA-CSIC-UIB), Esporles, Spain

**Correspondence:** Carmen Bouza (mcarmen.bouza@usc.es)

**Received:** 24 September 2024 | **Revised:** 11 February 2025 | **Accepted:** 28 February 2025

**Funding:** This work was supported by The Spanish Autonomous Agency of National Parks of the Ministry of Agriculture, Food and Environment.

**Keywords:** conservation | genetic diversity | genetic structure | microsatellites | mitochondrial haplotypes | pipefish | seahorses | species identification | Syngnathidae

## ABSTRACT

Spanish National Parks (NPs) are protected areas for biodiversity conservation, including two Maritime–Terrestrian NPs: The Atlantic Islands of Galicia, PNIA (NW Spain) and Archipelago of Cabrera, PNAC (Balearic Islands). This study was aimed to conduct a 3-year genetic survey of syngnathid fish species (i.e. seahorses and pipefish) identified in both NPs and nearby unprotected areas, using mitochondrial and microsatellite markers. A diversity of species was identified with differential distribution among NPs and adjacent areas studied. Pipefish (*Syngnathus acus*, *S. abaster, S. typhle*, *Entelurus aequoreus, Nerophis lumbriciformis*, *N. maculatus*, and *N. ophidion*) predominated, while seahorses (*Hippocampus guttulatus*) were much less abundant. Genetic data and phylogenetic analysis clarified in situ morphological identification. Mitochondrial haplotypes for each species clustered into monophyletic groups, supporting the identification of a cryptic lineage of *S. abaster* in PNAC distinct from eastern Mediterranean populations of this species. Intraspecific genetic diversity was evaluated at spatial and temporal scale for population samples recorded during the survey period, providing valuable information for individual resampling traceability and delineating management units. Temporal stability in genetic diversity and gene flow with adjacent areas were observed for dominant species within each NP in the 3-year period studied. However, significant intraspecific differentiation was detected between populations identified in Atlantic and Mediterranean NPs. This study provides valuable reference genetic data for future monitoring and to identify distribution or research gaps for further studies towards the conservation of syngnathid populations in Spanish marine NPs, which serve as umbrella species for the preservation of vulnerable coastal ecosystems and habitats.

## 1 | Introduction

Syngnathids (Family Syngnathidae) are charismatic fish with characteristic morphology, cryptic behaviour and specialised life history traits, including parental care and male pregnancy (Stolting and Wilson 2007; Kuiter 2009; Roth et al. 2020). Moreover, many syngnathid species face threats due to habitat degradation and disturbances due to human activities, overexploitation and bycatch and climate change (Vincent et al. 2011; Monteiro et al. 2023; IUCN 2024). In this

Manuel Vera and Belén G. Pardo contributed equally to this work, and they are first co-authors.

context, syngnathids may serve as valuable flagship species for the conservation of coastal vegetated habitats and ecosystems of special protection, including macroalgae communities and seagrass meadows (Stolting and Wilson 2007; Waycott et al. 2009; Planas et al. 2021; Peiffer et al. 2024). Thus, understanding their biology is crucial for defining conservation status and implementing protection measures for them and their associated ecosystems and habitats.

Sixteen syngnathid species are known to be distributed in European waters (Monteiro et al. 2023), of which 13 (two seahorses and 11 pipefishes) inhabiting the Spanish coasts (Planas et al. 2021; Castejón-Silvo et al. 2023). Most of them are classified as data deficient with unknown population status (IUCN 2024). This highlights the need for further research at distribution and population level, with focus on specific conservation characteristics and habitats at regional scale (Garner et al. 2020; IUCN 2024; https://www.iucn-seahorse.org/). Furthermore, seahorse species are listed as endangered by the Convention on International Trade of Wild Fauna and Flora (CITES 2024) and protected regionally in various European countries, including Spain, being assessed as near threatened in Mediterranean habitats (OSPAR 2008; Pierri et al. 2021; IUCN 2024), along with some pipefish in Balearic Islands (i.e. *Syngnathus abaster* and *S. typhle*; Planas et al. 2021).

Molecular genetic analysis is a valuable tool to assess taxonomic and evolutionary issues, and for evaluating genetic diversity at spatial and temporal scales to define conservation units (Allendorf et al. 2013; Garner et al. 2020), as applied in different syngnathids (i.e. Woodall et al. 2015; Montes et al. 2018; Mkare et al. 2021; Weiss et al. 2022). Mitochondrial DNA markers have been particularly useful to identify syngnathid species when morphological discrimination is ambiguous, as previously reported in European ranges (López et al. 2010; Hablützel and Wilson 2011; Sogabe and Takagi 2013; Woodall et al. 2018). Microsatellite loci have also shown high informative potential for conservation genetics studies in this group of fish (Luo et al. 2015; Endo et al. 2018; Wilson et al. 2021), including some European species, with limited sets of samples from Iberian Peninsula (*Hippocampus guttulatus* and *H. hippocampus*; López et al. 2010, 2012, 2015; *S. abaster*, Diekmann et al. 2009; Hübner et al. 2013; *Nerophis lumbriciformis*, Monteiro et al. 2014, 2017; *S. typhle*, Wilson and Veraguth 2010). Insufficient molecular genetic data is still revealed for most syngnathids distributed in Spanish coasts, particularly deficient in marine protected areas where suitable habitats have been identified for these species (Planas et al. 2021).

Marine National Parks (NPs) are highly valuable IUCN category II protected areas to preserve marine ecosystems and habitats, where deep characterisation of biodiversity components is required, including genetic resources of iconic species and evaluation of connected unprotected areas to assess coordinated management if needed (Planas et al. 2021; Stump et al. 2023). In Spain, there are two Maritime-Terrestrial NPs, which comprise two protected groups of islands and surrounding marine reserves: the Atlantic Islands of Galicia (PNIA; Atlantic Ocean, NW Spain) and the Archipelago of Cabrera (PNAC, Balearic Islands, Mediterranean Sea). These NPs contain valuable coastal habitats with vulnerable plant communities essential for various fish and mollusc species (Planas et al. 2021). In such context, syngnathid fish, as emblematic species, can serve as indicators of the condition of ecosystems with which they are associated, towards the management and conservation of marine biodiversity in coastal habitats (Gilby et al. 2017). However, knowledge of syngnathid fish biology, distribution and abundance in both Spanish marine NPs is very scarce, especially at the genetic level (Planas et al. 2021; Peiffer et al. 2024).

This study was framed within the first multidisciplinary conservation approach for studying syngnathids in the two Spanish marine NPs, which have different environmental characteristics and biodiversity profiles (Planas et al. 2021). The pioneering 1-year field analysis, based on a limited number of specimens, identified different syngnathid species in PNIA and PNAC using mitochondrial sequence data. These data represent a first baseline for genetic monitoring of species and populations in the Spanish NPs, beside putative connections to nearby unprotected areas (Planas et al. 2021), which remains fully unexplored.

In this context, this work aimed to address a genetic characterisation of syngnathids collected during a 3-year monitoring survey in both NPs and their adjacent unprotected areas. Mitochondrial and microsatellite markers were adjusted for the various species identified in the field sampling. Specific marker panels were first applied to validate species identification based on in situ morphological analysis. Subsequently, we assessed intraspecific genetic diversity and differentiation at spatial and temporal scales, exploring population connections with unprotected areas nearby the NPs. The biodiversity monitoring datasets were integrated to define conservation units and support guidelines for preserving syngnathids associated with vulnerable coastal habitats and ecosystems within marine protected areas.

## 2 | Material and Methods

### 2.1 | Biological Sampling

Samples were collected with permission from the Spanish Autonomous Organism of National Parks (OAPN), and animal handling procedures followed the European Ethics Committee (Planas et al. 2021). Thus, fish capture, handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (R.D. 1201/2005, 10th October) and the Regional Government Xunta de Galicia (Reference REGA ES360570202001/16/FUN/BIOL.AN/MPO02).

Genetic sampling was carried out between 2016 and 2018 during periodic field surveys at selected sites identified for syngnathids on the eastern coasts of the Cíes Islands in PNIA (42°13′N, 8°54′W; NW Spain; Atlantic Ocean) (Planas et al. 2021) and the Western coast of Cabrera Island in PNAC (39°08′N, 2°56′W; Balearic Islands, Mediterranean Sea) (Figure 1). Nearby unprotected marine areas in the Vigo Estuary in NW Spain (42°13′N, 8°48′W) and the Majorca Island (39°21′N, 2°41′W; Balearic Islands) (Figure 1) were also sampled to explore their population connectivity with the NPs. In PNIA, sampling site selection was based on previous knowledge on habitat suitability (i.e. seaweed, substrate, open water exposure), and located near the coastline (3–20-m depth in 2016; 2–15 m in 2017–2018) (Planas et al. 2021; Planas 2022). At least two diurnal underwater visual census

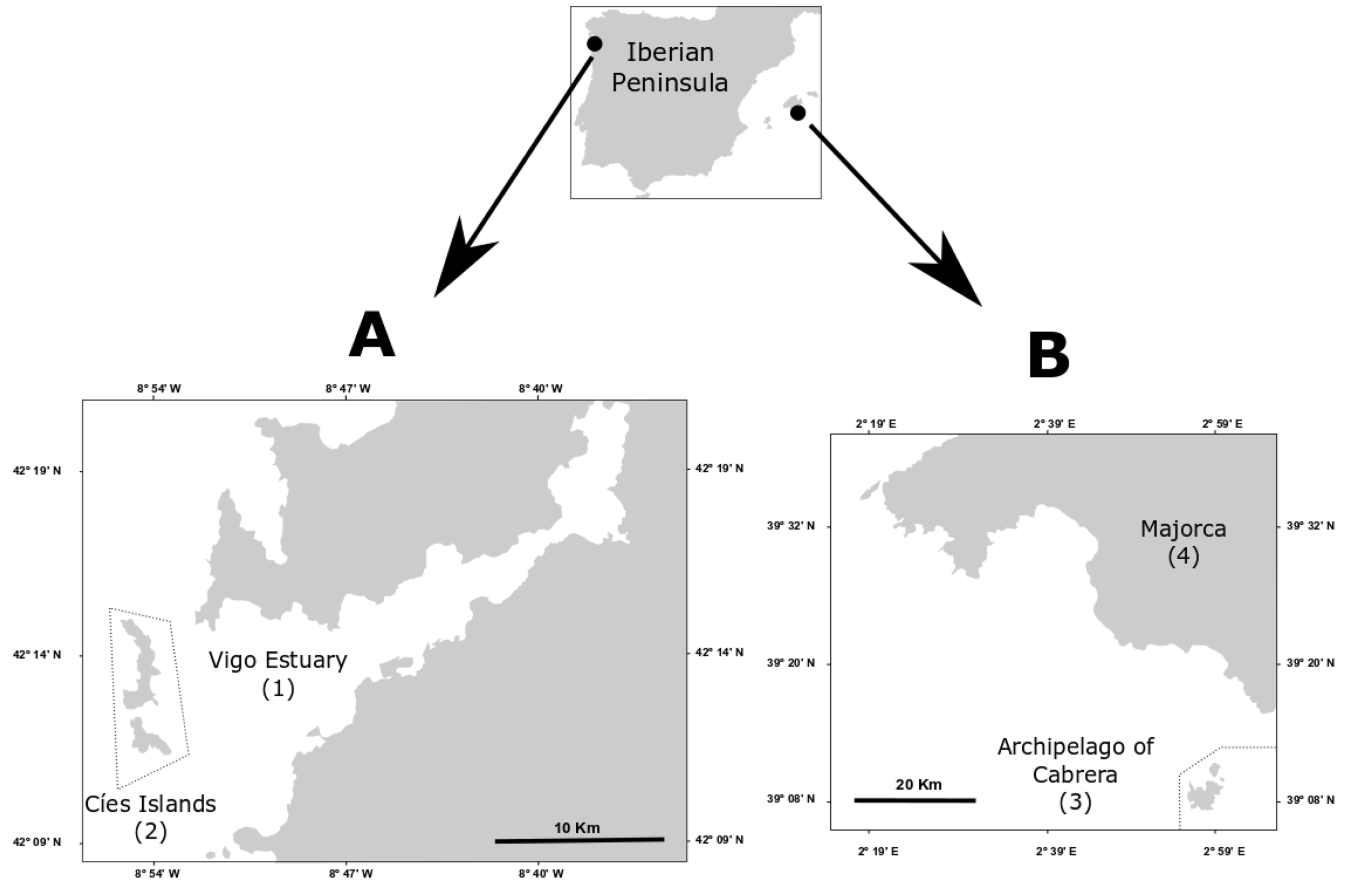


**FIGURE 1** | Location map of studied areas in Spanish Maritime-Terrestrial National Parks (NPs) of Atlantic Islands of Galicia, PNIA (A), and Archipelago of Cabrera, PNAC (B), and nearby unprotected areas. NP protected areas are included within doted polygons. The number between brackets identifies sampling codes for each sampling site, as shown in Table 1.

(UVC) surveys per site and season each year (spring, summer and winter) (50 min per survey) were conducted by two pairs of divers, who recorded and sampled the syngnathids sighted. In PNAC, sampling sites were selected in suitable benthic habitats covered by seagrass and surveyed by two pairs of divers (two surveys per site, 60–80 min per survey; 2.8–21.5-m depth gradient; Planas et al. 2021). Due to the low number of sightings by UVC during the first year, a traditional small trawl net (called *ganguil*; 3-m-long, 0.8-m mouth aperture, 1.2-cm$^2$ mesh size) was used for sampling sets in PNAC and Majorca (7 to 9 days per site and year; net operating depth: 11 to 16.5 m, under permission by park authorities; Planas et al. 2021). A rolling stainless-steel cylinder was incorporated in the bottom of the net mouth to protect seagrass species from physical damage during sampling (i.e. *Cymodocea nodosa*), being only authorised in selected site meadows avoiding disturbance of endemic species (i.e. fan mussel, *Pinna nobilis;* Planas et al. 2021).

For each specimen, a small portion of tissue was taken from the dorsal fin (nonlethal fin-clipping) and preserved in 95% ethanol for subsequent genetic analyses, and alive specimens subsequently released. Instead, a small portion of muscle tissue was taken in very small specimens from PNAC. Each of the four sampling areas was assigned a number (i.e. (1) Vigo Estuary; (2) PNIA; (3) PNAC; (4) Majorca) for easier identification among species, which were coded with initials to distinguish genus and species names (e.g. SA for *S. acus*; SAB for *S. abaster*; Table 1).

A collection of 418 specimens was genetically analysed across the marine study areas, with species identification based on visual morphology during field surveys (Planas et al. 2021). Several seahorse and pipefish species were identified (Table 1), supported by genetic analysis in this study, except for a few discordant samples (see Results). Genomic DNA extraction protocols were adjusted for small quantity of sampled tissues, using NucleoSpin Tissue XS kit (Macherey–Nagel) and, when necessary, amplified using GenomiPhi V2 kit (GE Healthcare) always following manufacturers' recommendations. DNA concentrations (in ng/μL) for each extraction were measured with NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific).

## 2.2 | Mitochondrial DNA Analyses

Cytochrome b (Cytb) and 16S ribosomal DNA (16S-rDNA) markers were used for syngnathid phylogenetic and population studies (Wilson et al. 2001; López et al. 2010). Universal Cytb primers, L14275F (Pääbo et al. 1991) and H15926R (Wilson et al. 2001) were used in pipefish of the genera *Syngnathus* and *Entelurus* (Braga-Gonçalves et al. 2017). Specific Cytb primers were used for *H. guttulatus* (SHORSE5.3L, Casey et al. 2004; GUTCYTBR, Woodall et al. 2015), also assayed in reference DNA samples of this species from Cantabrian (CA) and Atlantic (AT) coasts in NW Spain (López et al. 2015). Due to failed Cytb

**TABLE 1** | Sampling of syngnathids in the National Parks of Atlantic Islands (PNIA) and Archipelago of Cabrera (PNAC), and nearby unprotected areas (Vigo Estuary and Majorca Island). *N*: sampling size; nd: no sampled collection in these years. -: no samples detected during field surveys. Number between brackets identifies each sampling site (1: Vigo Estuary; 2: PNIA; 3: PNAC; 4: Majorca). Number between parentheses represents the individuals collected within the National Parks (NPs).

| Locations/ Species | Code | 2016 | 2017 | 2018 | *N* |
|---|---|---|---|---|---|
| Vigo Estuary (1) | | | | | 109 |
| *Syngnathus acus* | SA1 | 41 | nd | nd | |
| *Syngnathus typhle* | ST1 | 33 | nd | nd | |
| *Nerophis lumbriciformis* | NL1 | 35 | nd | nd | |
| PNIA (2) | | | | | 192 |
| *Syngnathus acus* | SA2 | 22 | 70 | 82 | |
| *Hippocampus guttulatus* | HG2 | 4 | 6 | — | |
| *Entelurus aequoreus* | EA2 | — | 4 | 4 | |
| PNAC (3) | | | | | 71 |
| *Syngnathus abaster* | SAB3 | 6 | 10 | 22 | |
| *Syngnathus typhle* | ST3 | — | 13 | 6 | |
| *Nerophis maculatus* | NM3 | 1 | 1 | 5 | |
| *Nerophis ophidion* | NO3 | — | 1 | 6 | |
| MAJORCA (4) | | | | | 46 |
| *Syngnathus typhle* | ST4 | nd | 13 | 11 | |
| *Nerophis maculatus* | NM4 | nd | 2 | 15 | |
| *Syngnathus abaster* | SAB4 | nd | — | 2 | |
| *Syngnathus acus* | SA4 | nd | — | 2 | |
| *Hippocampus guttulatus* | HG4 | nd | — | 1 | |
| Total (NPs) | | | | | 418 (263) |

amplification in *Nerophis* pipefish samples, 16S-rDNA was analysed using universal primers (16Sa-L2510, 16Sb-H3080; Palumbi et al. 1991). Cytb and 16S-rDNA amplification was adjusted in 50 μL reactions: 75–100 ng of DNA, 1× buffer, 2.5 mM MgCl2, 400 μM dNTPs, 0.2 μM primer, 1 U Taq polymerase (Bioline AgroScience for Cytb; Applied Biosystems for 16S-rDNA), except in *H. guttulatus* (1.25 U Taq polymerase). The PCR program consisted of 95°C for 5 min, followed by 33 cycles of 93°C for 1 min, 50°C for 1 min (48°C in *Entelurus aequoreus*), 72°C for 3 min and a final extension at 72°C for 10 min. Specific conditions were used for *H. guttulatus* (94°C, 2 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min and 72°C, 2 min). Amplified fragments were verified on 1.5% agarose gels and subsequently purified using Exo-SAP (USB). Sequencing was performed with BigDye™ Terminator v3.1 Cycle Sequencing, following manufacturer's recommendations, on an ABI 3730 XL automatic sequencer (Applied Biosystems). Additionally, 33 previously sequenced specimens from preliminary PNIA and PNAC surveys in 2016 were also included (Planas et al. 2021).

Manual sequence review and detection of variable nucleotide positions was performed with SEQSCAPE 4.0 (Applied Biosystems), using Cytb and 16S-rDNA reference sequences of Northeast Atlantic syngnathids deposited in GenBank (see Results). Multiple sequence alignment and editing were performed using BIOEDIT 7.0 (Hall 1999) with its 'ClustalW multiple alignment' module, applying the default parameters for the alignments. Haplotypes were detected with DNASP 5.10 (Librado and Rozas 2009). Variable position files and phylogenetic reconstructions were obtained with MEGA 7.0 (Kumar et al. 2016), using the neighbour-joining (NJ) method with p-distance as nucleotide substitution rate model for all the species and the significance of nodes tested by bootstrapping (1000 replicates). Population sequence variation for each species was analysed using the number of haplotypes (H), haplotype (h) and nucleotide ($\pi$) diversity, and the interpopulation differentiation component ($\Phi_{ST}$) (applying the proper nucleotide substitution rate [i.e. TN93 with gamma value of 0.23 for *Syngnathus* species and T92 with gamma value of 0.37 for *Nerophis* species]) using ARLEQUIN 3.5 (Excoffier and Lischer 2010). The haplotypes previously detected for 33 specimens of four syngnathid species identified in the NPs in 2016 (22 *S. acus* and four *H. guttulatus* in PNIA; six *S. abaster* and one *N. maculatus* in PNAC; Planas et al. 2021) were integrated with the 385 newly analysed samples in this study, 109 of the collected in 2016.

## 2.3 | Microsatellite Analyses

Specific microsatellite panels per species were adjusted using primers reported for different syngnathids to analyse all samples studied from 2016 to 2018 (Table 1). In seahorses, 12 loci were assayed, following previous protocols (López et al. 2015) (see Table S1, summarizing loci information used for all syngnathid species). In pipefish, 19 loci isolated from different European distributed species were tested in this study: *S. typhle* (Styph12, Wilson and Veraguth 2010; Syty4, 16–18, 22, 24, Roth et al. 2012), *S. abaster* (Sabas3, 5, 7–9; Diekmann et al. 2009) and *N. lumbriciformis* (NL00629, NL00750, NL02383, NL05025, NL06490, NL07690, NL12901 and NL16858; Monteiro et al. 2014). To optimise the informative potential across pipefish species, based on technical, polymorphism and allelic range criteria, different subsets of loci were adjusted in multiplex reactions (Table S1). The 10-μL PCR

reactions included 30 ng of DNA, 1× 'Master Mix kit Go Taq G2 Hot Start Colorless' (Promega) and specific primer concentration (shown in the Table S1). PCR conditions included 95°C, 2 min; 25 cycles (30 in *E. aequoreous*, *N. maculatus* and *N. ophidion*) of 94°C for 30 s, specific annealing temperature (see values in Table S1) for 90 s, and 72°C for 1 min; and final extension at 72°C, 5 min.

Microsatellite markers were genotyped using the ABI PRISM® 3730xl automated sequencer and GeneMapper 4.0 (Applied Biosystems). Genotyping reliability and presence of null alleles were evaluated with MICROCHECKER (Oosterhout et al. 2004). Hardy–Weinberg (HW) equilibrium and linkage disequilibrium (LD) between all loci pairs per population and overall were tested using GENEPOP 4.04.7 (Rousset 2008), applying Bonferroni correction. Genetic diversity per locus and population was estimated using FSTAT 2.9.3.2 (Goudet 2001): number of alleles per locus (A), allelic richness (AR) and expected (He) heterozygosity. FSTAT was also used to estimate the fixation index ($F_{IS}$). The global and pairwise interpopulation genetic differentiation component ($F_{ST}$) was estimated by FSTAT, using 5000 iterations. Intraspecific genetic structure was analysed using STRUCTURE 2.3.4 (Pritchard et al. 2000), exploring a number of clusters ($K$) between 1 and the number of population samples plus 1, with 10 replicates per K (30,000 burn-in cycles, 80,000 iterations). Probabilities of $K$ values were analysed using STRUCTURE HARVESTER 0.6.94 (Earl and Vonholdt 2012), and cluster integration for different $K$ was performed using CLUMPAK (Kopelman et al. 2015). For *H. guttulatus* from PNIA, individual assignment analysis to reference populations from NW Spain could be performed based on previously reported genotypes at common microsatellite loci (López et al. 2015) using GENECLASS2.0 (Piry et al. 2004), applying the Rannala and Rannala and Mountain (1997) criterion for likelihood estimation, and the simulation algorithm of Paetkau et al. (2004) to estimate the assignment probability for each seahorse to each reference population.

## 3 | Results

### 3.1 | Mitochondrial Variability and Phylogenetic Relationships

The sequencing of mitochondrial DNA (mtDNA) markers supported the visual morphological analysis for species identification. The genetic sampling in the study areas throughout the survey supported the scarce presence of long-snouted seahorses (*H. guttulatus*; subfamily Hippocampinae) and more diversity of pipefish species from two divergent subfamilies (Hamilton et al. 2017): Syngnathinae (*S. acus*, *S. typhle* and *S. abaster*) and Nerophinae (*N. lumbriciformis*, *N. maculatus*, *N. ophidion*, *E. aequoreus*) (Table 1).

In pipefish of the genus *Syngnathus*, a fragment of the Cytb gene of 1149 base pairs (bp) was analysed. In *S. acus* specimens found in PNIA-Cíes and Vigo Estuary, 40 haplotypes were detected, with 51 variable sites (see Figure S1). One haplotype (Cytb_SA13) was identical to GenBank reference AF356040 for this species from Sweden (Wilson et al. 2001), used for the alignments of *S. acus* and *S. typhle*. The most frequent haplotypes in PNIA-Cíes (Cytb_SA02, Cytb_SA14) were common in the nearby Vigo Estuary, although rare haplotypes were also found (see Table S2A for spatial haplotype distribution and frequency). In *S. typhle*, 39 haplotypes with 55 variable sites were detected (Figure S1), with no shared haplotypes between NPs (see Table S2A). Cytb_ST05 and Cytb_ST21 were the most frequent in PNIA and PNAC, respectively, although many haplotypes (30) were rare in both NPs. In all *S. abaster* specimens analysed in PNAC and Majorca Island, a total of eight haplotypes (Cytb_SAb01–Cytb_SAb08) with 23 variable sites were identified (Figure S1), including the two previous haplotypes reported in the first year of the field survey (Cytb_SAb1 and Cytb_SAb2; Planas et al. 2021). Cytb_SAb01 was identical to that detected in two specimens initially misidentified as *S. rostellatus* based on visual morphology in PNAC (Cytb_SR01), which were later reclassified as *S. abaster* (see Table S2B, summarizing all misidentifications detected). These misclassifications were collected in the first year of sampling, highlighting the importance of caution in visual identifications for these species especially in small individuals and poorly trained teams. The phylogenetic analysis revealed monophyletic groups for each identified *Syngnathus* species, with *S. abaster* more closely related to *S. typhle* (Figure 2). The *S. abaster* haplotypes in PNAC clustered separately from GenBank sequences of this species from Italy (Mwale et al. 2013) and other Mediterranean-distributed pipefish species, although closer to *S. typhle* and *S. taenionotus* than to *S. acus* and *S. rostellatus* (Figure 2).

In the genus *Nerophis*, the 16S-rDNA sequence of *N. ophidion* (GenBank AF354994) was used to align a 521-bp fragment with 95 variable positions and three indels (see Figure S2). Four haplotypes (16S_NL01-16S_NL04) were detected in *N. lumbriciformis* from Vigo Estuary. In *N. maculatus* from PNAC and Majorca, five haplotypes were found, with 16S_NM01 being the most abundant (see Table S2A for details on haplotype frequency across sampling areas). A few morphological misclassifications were detected (Table S2B): One *N. maculatus* in PNAC was genetically reclassified as *N. ophidion*, highlighting again the challenges of visual morphological discrimination, especially for small-sized pipefish species with poorly differentiated morphological features. The most abundant haplotype for the specimens genetically identified as *N. ophidion* was 16S_NO02, identical to the reference AF354994 from Sweden (Wilson et al. 2001). The phylogenetic tree grouped the haplotypes of the three *Nerophis* species into three monophyletic clades (Figure S3).

In *E. aequoreus*, found only in PNIA, three haplotypes for the 565-bp 16S-rDNA fragment with two variable positions were detected (Figure S2). The most abundant haplotype, 16S_EA01, was identical to the Norwegian reference used for this species (KY065539; Hamilton et al. 2017). In addition, for a 900-bp Cytb aligned fragment with respect to the GenBank KY857646 sequence from the European Atlantic coast (Braga-Gonçalves et al. 2017), seven haplotypes with 15 variable positions were detected, with Cytb_EA01 being the most abundant (Figure S2).

Six haplotypes of *H. guttulatus* were identified using a Cytb fragment of 507 bp, integrating with other available haplotypes from the Galician coast, revealing a total of 16 haplotypes and 16 variable positions, aligned against the GenBank AF192664 sequence from UK (Casey et al. 2004) (Figure S4). Cytb_HG16 was

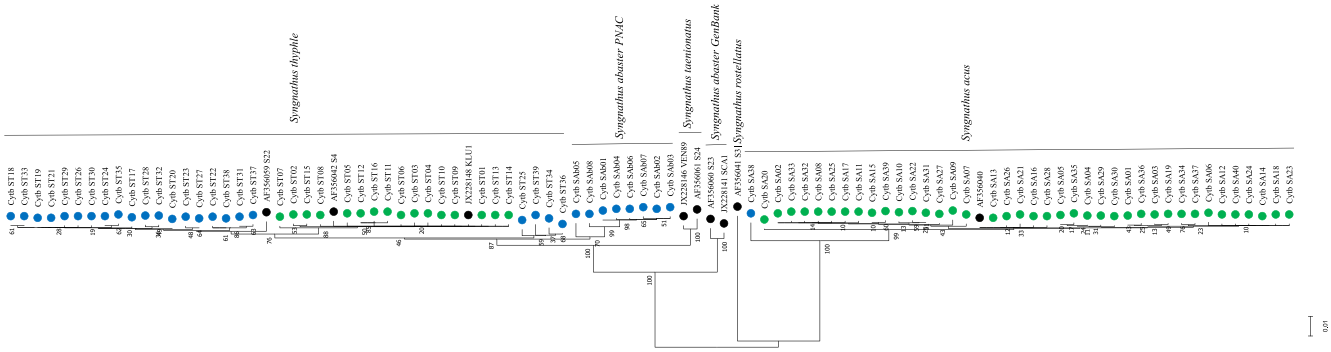


**FIGURE 2** | NJ (neighbour-joining) tree showing the relationships among the Cytochrome b (Cytb) haplotypes detected in species of Genus *Syngnathus* in the Spanish National Parks with respect to reference sequences of congeneric European species (Mwale et al. 2013): *S. abaster* (AF356060_S23; JX228141_SCA1), *S. typhle* (AF356042_S4; AF356059_S22; JX228148_KLU1), *S. acus* (AF356040), *S. rostellatus* (AF356041_S3) and *S. taenionatus* (AF356061_S24; JX228146_VEN89). Numbers in nodes show bootstrap values (1000 replicates) for robustness of branches. Green and blue colours correspond to the presence in PNIA and PNAC, respectively, while black colour identifies the reference sequences.

unique in PNAC, whileCytb_HG03 was the most common in PNIA and NW Spain (CA and AT, Table S2A). A putative *H. hippocampus* in PNAC was genetically reclassified as *H. guttulatus*, suggesting a possible morphological confusion between both species, as reported in Gran Canaria Island (López et al. 2010). Phylogenetic reconstruction identified a monophyletic group for *H. guttulatus* separated from the congeneric species *H. hippocampus* also presents in Galician coasts (Valladares et al. 2014; see Figure S5).

Morphological analysis combined with genetic samples identified 217 *S. acus*, 40 *S. abaster*, 76 *S. typhle*, 11 *H. guttulatus*, 8 *E. aequoreus*, 35 *N. lumbriciformis*, 24 *N. maculatus* and 7 *N. ophidion* specimens in the temporal period studied (Table 1). All new haplotypes were submitted to GenBank database (for detailed accessions information, see Table S2A).

### 3.2 | Microsatellite Adjustment for Pipefish Species

In pipefish species, multiplex PCR conditions were adjusted for selected informative microsatellite subsets per species (see Table S1 for a summary of the species-specific marker panels and conditions), ranging from seven to nine loci in *Syngnathus* species, and from five to eight loci in Nerophinae pipefish. In Nerophinae, especially *N. ophidion*, low cross-amplification rate, genotyping success (e.g. NL02383 and NL05025) and polymorphism (NL00629) were observed starting from loci isolated in *N. lumbriciformis* (Monteiro et al. 2014).

The variation of microsatellite genotypic data in each pipefish species (displayed in Table S2C) showed global adjustment to HW equilibrium, except for some loci exhibiting heterozygote deficit suggestive of null alleles, with caution due to low sample size in some species. No significant LD was detected between pairs of loci, compatible with independent segregation.

### 3.3 | Population Genetic Diversity and Structure

Diversity and population genetic structure per species were estimated based on mtDNA and microsatellite markers (Table 2), allowing short-term temporal comparisons for the best represented species in the sampling period studied.

*Sygnathus acus* was the predominant species recorded in PNIA and the Vigo Estuary, with limited presence in Majorca Island (Table 2). Genetic diversity estimates were similar among temporal samples in PNIA compared with the Vigo Estuary (Table 2). Low and non-significant genetic differentiation was observed between spatial and temporal samples of *S. acus* in PNIA and nearby areas (overall $F_{ST}$ and $\Phi_{ST}$: ~2%; mean paired $F_{ST}$ and $\Phi_{ST}$: 1.6% and 2.2%, respectively) (see Table S3, summarizing all pairwise $F_{ST}$ and $\Phi_{ST}$ estimates in each species). Significant differentiation was only found between SA2-16 and SA2-18 with mtDNA, potentially due to unequal sample sizes (larger in SA2-18; Table 1) and the smaller effective size for haploid mitochondrial genome (than nuclear one). STRUCTURE analysis identified the best number of clusters as $K=2$, followed by $K=3$, based on Evanno's Delta K values (Evanno et al. 2005). The observed pattern revealed a genetically homogeneous group comprising all individuals from PNIA and the Vigo Estuary, while the few Majorca samples showed differentiated ancestry proportions based on microsatellite loci (see structure plots in Figure S6), agreeing with mtDNA data for *S. acus* (Figure 2), of interest to be confirmed in further Mediterranean surveys based on higher sample sizes.

In *S. typhle* identified in PNAC, Majorca and Vigo Estuary, no significant differences in genetic diversity levels were detected between spatial or temporal samples (Table 2). Significant microsatellite and mtDNA estimates of differentiation were observed between Atlantic and Mediterranean populations (mean pairwise $F_{ST}$: 7.3%; $\Phi_{ST}$: 68.9%; $p<0.05$; Table S3), which, beyond influence of some small/unequal samples sizes, were highly consistent with mtDNA phylogeny data for *S. typhle* (see Figure 2). Low and non-significant differentiation was detected between spatial and temporal sample pairs from PNAC and Majorca (global and mean pairwise $F_{ST}$ values: ~0.1%; $\Phi_{ST}$: 4%; Table S3; $F_{ST}$ of 0.02% and $\Phi_{ST}$ of 2.9%, when excluding lower/unequal sample sizes ($N<10$)), except between ST3-17 and ST4-18 with mtDNA, likely due to low sample size. Genetic structure analysis revealed divergent Atlantic and Mediterranean populations, grouping all PNAC

**TABLE 2** | Genetic diversity of syngnathid fish in the Spanish National Parks and adjacent areas (codes are in Table 1).

| *Species* | Mitochondrial DNA* | | | | Microsatellites | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Population** | **N** | **H** | ***h*** | ***π*** | **N** | **A** | **AR** | **He** | **$F_{IS}$** | **Loci used** |
| *S. acus* | | | | | | | | | | 9 |
| SA1-16 | 35 | 14 | 0.866 | 0.0030 | 40 | 10.44 | 9.12 | 0.620 | 0.018 | |
| SA2-16 | 22 | 9 | 0.779 | 0.0029 | 22 | 8.78 | 8.78 | 0.623 | 0.036 | |
| SA2-17 | 70 | 25 | 0.865 | 0.0026 | 69 | 11.11 | 8.62 | 0.614 | 0.030 | |
| SA2-18 | 82 | 17 | 0.831 | 0.0023 | 82 | 11.56 | 8.68 | 0.658 | 0.050 | |
| SA4-18 | 2 | 1 | 0.000 | 0.0000 | 2 | 1.89 | — | 0.556 | 0.500 | |
| *S. typhle* | | | | | | | | | | 9 |
| ST1-16 | 28 | 15 | 0.931 | 0.0028 | 32 | 15.56 | 5.54 | 0.716 | 0.079 | |
| ST3-17 | 12 | 9 | 0.939 | 0.0035 | 12 | 8.89 | 5.69 | 0.761 | 0.097 | |
| ST3-18 | 6 | 6 | 1.000 | 0.0063 | 6 | 6.11 | 5.54 | 0.744 | 0.144 | |
| ST4-17 | 13 | 8 | 0.859 | 0.0019 | 13 | 9.44 | 5.80 | 0.768 | 0.102 | |
| ST4-18 | 11 | 6 | 0.727 | 0.0050 | 11 | 8.56 | 5.71 | 0.782 | 0.143 | |
| *S. abaster* | | | | | | | | | | 7 |
| SAB3-16 | 6 | 2 | 0.533 | 0.0005 | 6 | 6.57 | 5.93 | 0.767 | 0.081 | |
| SAB3-17 | 10 | 3 | 0.378 | 0.0005 | 10 | 8.14 | 5.69 | 0.829 | 0.139 | |
| SAB3-18 | 21 | 5 | 0.810 | 0.0014 | 21 | 11.14 | 5.38 | 0.746 | 0.016 | |
| SAB4-18 | 2 | 2 | 1.000 | 0.0159 | 2 | 3.71 | — | 1.000 | 0.143 | |
| *H. guttulatus* | | | | | | | | | | 12 |
| HG2-16/17 | 9 | 5 | 0.667 | 0.0020 | 9 | 5.50 | 5.50 | 0.632 | 0.004 | |
| HG4-18 | 1 | 1 | — | — | 1 | — | — | — | — | |
| HG-CS[a/b] | 27[a] | 9 | 0.749 | 0.0021 | 52[b] | 10.50 | 5.58 | 0.572 | 0.002 | |
| HG-SA[a/b] | 38[a] | 12 | 0.760 | 0.0022 | 51[b] | 11.42 | 5.85 | 0.610 | 0.052 | |
| *N. lumbriciformis* | | | | | | | | | | 8 |
| NL1-16 | 31 | 4 | 0.187 | 0.0004 | 35 | 10.75 | — | 0.769 | 0.024 | |
| *N. maculatus* | | | | | | | | | | 5 |
| NM3-16/18 | 7 | 3 | 0.524 | 0.0017 | 6 | 4.60 | 2.68 | 0.705 | 0.270 | |
| NM4-17/18 | 17 | 9 | 0.735 | 0.0020 | 16 | 9.20 | 2.93 | 0.723 | **0.268** | |
| *E. aequoreus* | | | | | | | | | | 8 |
| EA2-17/18 | 8 | 7 | 0.964 | 0.0049 | 8 | 5.38 | — | 0.545 | 0.388 | |

*Cytb marker in Genera *Syngnathus* and *Hippocampus*, while 16S-rDNA in *Nerophis* and *Entelurus*. *N*: Sample size (samples with very low *N* (< 5) were merged with consecutive temporal replicates and the year interval was indicated in the code; e.g. HG2-16/17). H: haplotype no., *h*/*π*: haplotype/nucleotide diversity; A: allele no., AR: allelic richness, He: expected heterozygosity; FIS: fixation index (bold: $P < 0.05$);[a/b] *H. guttulatus* reference samples from Cantabrian (HG-CS) and Atlantic (HG-SA) coasts in NW Spain ([a]Cytb data analysed in this study; [b] previously reported microsatellite data in HG-CS and HG-SA from Betanzos and Vigo Estuary, respectively; López et al., 2015). All available samples per species were presented, more reliable diversity estimates the larger the sample sizes. For detailed information about the microsatellite loci used in each species, see Table S1.

and Majorca samples, for different *K* values, with the highest Delta K for $K = 3$ (LnP: −3188.82) (Figure 3).

In *S. abaster*, detected in PNAC and Majorca, the Cytb haplotype analysis supported its morphological classification as a monophyletic group, suggesting a cryptic lineage distinct from other eastern Mediterranean *S. abaster* populations, with a greater genetic distance compared with other congeneric species (Figure 2), as previously indicated by preliminary limited sampling in 2016, during the first year of field survey (Planas et al. 2021). Microsatellite genetic diversity within the whole population data set in PNAC and Majorca, using loci isolated from *S. abaster* (Diekmann et al. 2009), was also consistent with intraspecific variation, showing no signs of mixing between genetically heterogeneous populations (i.e. neither deviations from HW equilibrium nor significant LD between loci

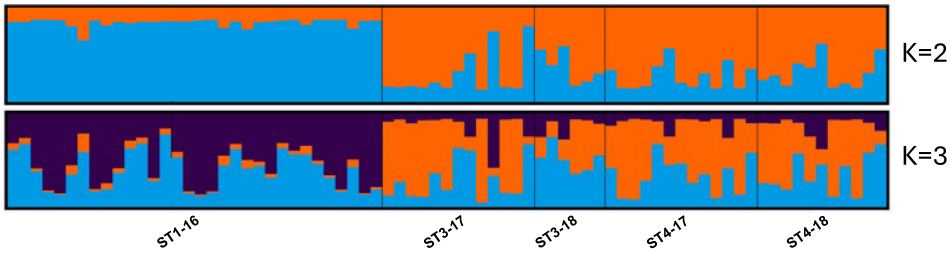


**FIGURE 3** | Genetic structure of *Syngnathus typhle* in the National Parks and adjacent areas. Populations are separated by vertical lines, and each specimen is represented by individual bars with their colour proportion representing the posterior probability of assignment of each individual to the different clusters (K) tested. This result was estimated by the STRUCTURE program, which demonstrates the results for $K = 2$ and $K = 3$.

pairs per population or globally in the samples analysed were detected). Microsatellite allelic diversity and richness were similar between temporal samples in PNAC, although higher in SAB3-18 with mtDNA, likely influenced by smaller sample sizes in 2016 and 2017 (Table 2). No significant microsatellite differentiation was detected (global $F_{ST}$: 1.5%, $p > 0.05$; $F_{ST}$ of 3.1% when excluding small samples with $N < 10$; see all pairwise estimates in Table S3). In contrast, mtDNA data showed significant differentiation ($\Phi_{ST}$: 30%, excluding samples with $N < 10$) which was highly influenced by rare haplotypes under unequal sample sizes. Finally, using STRUCTURE with microsatellite markers identified a single cluster grouping all individuals of this cryptic *S. abaster* lineage detected in PNAC and nearby Balearic areas (LnP: −1203.50).

In *N. maculatus* from PNAC and Majorca, temporal analysis was conditioned by restricted and unequal sample sizes. Spatial clustering revealed slightly lower genetic diversity in PNAC compared with Majorca, with genetic diversity estimators least affected by sample size using both microsatellites and mtDNA (mean He, AR, $\pi$; Table 2). $F_{ST}/\Phi_{ST}$ values between PNAC and Majorca were small and nonsignificant with both marker types (below 0.1%; $p > 0.05$; Table S3); beyond sampling size influence, results were consistent with a single most likely population group (LnP: −387.42) in the genetic structure analysis, across K values.

In *N. lumbriciformis*, a single population sample was identified in Vigo Estuary, exhibiting lower mtDNA variability than the other *Nerophis* species with 16S-rDNA, while more similar microsatellite genetic diversity estimates (He: 0.768; A: 10.8; Table 2). Even so, caution is needed for interspecific comparisons based on specific subsets of microsatellite loci, under small/unequal sample sizes and should be revised in further studies.

For the remaining pipefish, *N. ophidion* from PNAC and *E. aequoreus* from PNIA, the low number for all genetic samples ($N < 10$) and smaller number of microsatellite subsets with low polymorphism, restricting comprehensive genetic analysis. In addition, some *N. ophidion* specimens in PNAC showed low DNA quality, with poor amplification success for some loci ($\leq 50\%$ for NL02383 and NL05025). Even so, multilocus analysis in this species allowed us to obtain preliminary genetic diversity estimates (He: 0.842; A: 4.8) and individual identification, discarding putative resampling, using the most informative loci (NL02383, NL1685, NL0769, with five, six, and nine alleles per locus, respectively). For *E. aequoreus* samples from PNIA, preliminary estimates of genetic diversity were obtained with microsatellites and mtDNA (He: 0.545; A: 5.4; H: 7; h: 0.964; Table 2).

Genetic samples of *H. guttulatus* in PNIA and Majorca were scarce. One Majorca specimen exhibited differential genetic variants compared with PNIA, while another could not be analysed due to poor DNA quality, aligning with sighting data (surface flotation). In PNIA, preliminary genetic diversity estimates were obtained with microsatellites and mtDNA (He: 0.632; A: 5.5; H: 5; h: 0.667; Table 2). In the genetic assignment analysis (see results in Table S4), all these seahorses were allocated to the Galician reference populations from Atlantic and Cantabrian estuaries previously studied using common microsatellites (López et al. 2015), and many specimens (67%) were assigned with high probability to the Vigo Estuary, nearby the Cíes Islands. This information is concordant with the mitochondrial Cytb data in this work, pointing to low genetic structure along the Galician coasts for this species, as reported (López et al. 2015). While being cautious with the low sample size, no signs of significant genetic differentiation were found from any of the comparisons using microsatellite data (mean $F_{ST}$: 0.1%; $\Phi_{ST}$: 0.9%; $p > 0.05$).

## 4 | Discussion

### 4.1 | Genetic Species Identification of Syngnathids

Morphological and genetic analysis identified eight syngnathid species in the Spanish marine NPs and nearby unprotected areas during 2016–2018, representing an important diversity out of the 16 species reported with European distribution (Monteiro et al. 2023). Compared with 2016 surveys (Planas et al. 2021), the expanded dataset increased species diversity (from five to seven) in both protected areas, highlighting the dominance of different pipefish in PNIA and PNAC, and the very low abundance of seahorses in NPs (*H. guttulatus* only detected in PNIA). *Hippocampus guttulatus* exhibits higher abundances in estuarine and sheltered coastal habitats in the NW Iberian coasts compared with other European areas (Valladares et al. 2014; Planas et al. 2021; Peiffer et al. 2024). The mtDNA analyses clarified some morphological misidentifications among some pipefish species (total 2.3%; eight in PNAC, two in Majorca), emphasizing the need for careful visual surveys and genetic analyses (López et al. 2010; Hablützel and Wilson 2011; Garcia et al. 2019). Distinct syngnathid species distribution was observed between PNIA and PNAC, associated with specific habitats, environmental and biodiversity profiles in Atlantic and Mediterranean areas, including different prevalence of seaweed

communities (Planas et al. 2021). PNIA featured two pipefish species, *S. acus* and *E. aequoreus,* in addition to the seahorse *H. guttulatus*, with low abundances for the latter two. *Syngnathus acus* was also dominant in nearby Vigo Estuary, where additional pipefish species were identified (*N. lumbriciformis* and *S. typhle*), agreeing with specific biodiversity patterns including estuarine seagrass meadows, which are absent in PNIA (Planas et al. 2021). In PNIA, sheltered suitable areas include complex seaweed communities and substrates (rocky, sandy-gravel and maerl beds), while benthic communities and seagrass meadows in the Balearic sites (Planas et al. 2021). In PNAC, four pipefish species were detected, with *S. typhle* and *S. abaster* being more prevalent and *N. maculatus* and *N. ophidion* occurring at much lower abundance. In the adjacent Majorca Island area, there was also presence of *S. acus* and *H. guttulatus*, while *N. ophidion* was absent, suggesting limited syngnathid diversity and low abundance in the sampled areas and associated habitats, which can be partly explained by the sampling methods used in the three-year period studied. Syngnathid species richness and abundance could have been underestimated due to fish crypsis in dense meadows during visual censuses, and to depth ranges imposed by net permissions in PNAC (11–16.5 m), which may not fully fit for some species, such as *S. abaster* (also inhabiting above 5 m) (Planas et al. 2021; Castejón-Silvo et al. 2023), worth of further temporal surveys to capture long-term genetic trends.

Finally, *S. abaster* was detected throughout the survey period in PNAC and Majorca Island, supporting preliminary results based on six specimens sighted in PNAC during 2016 (Planas et al. 2021). All *S. abaster* haplotypes in PNAC and nearby areas clustered into a monophyletic group, clearly differentiated from Cytb haplotypes of Italian *S. abaster* at eastern Mediterranean areas (Mwale et al. 2013). This suggests a divergent cryptic genetic lineage for this species in PNAC and Majorca Island, without morphological differences compared with those described in Italian coasts, suggesting historical evolutionary isolation processes in this pipefish. Future morphological and genetic surveys on less studied western Mediterranean populations of *S. abaster* are necessary to investigate this further (Sanna et al. 2013; Planas et al. 2021), as invoked for complex systematic relationships in other syngnathids (Garcia et al. 2019).

### 4.2 | Genetic Diversity and Structure: Management Units

This study presents the first population genetic diversity data for *S. acus* from PNIA and Vigo Estuary, where temporal stability in genetic diversity was observed. These populations form a single gene pool, indicating connectivity and, therefore, can be considered as a single genetic management unit. This connectivity may be associated with the shared use of food resources by the algal communities in both areas, with predominance of large mature breeders during the spawning season in PNIA, during the 3-year period surveyed, as reported (Planas et al. 2021; Planas 2022).

For *S. typhle*, from PNAC, Majorca and Vigo Estuary, although genetic diversity was similar between the areas, temporal stability was observed in PNAC in the period studied. Furthermore, significant genetic differentiation was detected between the Atlantic (Vigo Estuary) and Mediterranean (PNAC and Majorca) populations, consistent with previous phylogeographic studies (Wilson and Veraguth 2010).

For *S. abaster*, from PNAC and Majorca, we attribute it to a cryptic lineage distinct from other Mediterranean populations, which may be due to geographic isolation leading to a historic speciation event, as suggested (Sanna et al. 2013). Although microsatellite genetic diversity was comparable to Atlantic populations (Diekmann et al. 2009), we suggest that further research be conducted to assess whether our hypothesis holds.

Genetic diversity levels in other pipefish species (e.g. *Nerophis* and *Entelurus*) were influenced by low sample sizes at spatial and/or temporal scale in the period studied, which prevents us from providing definitive conclusions. Even so, some evidence and gaps can be derived from this study of interest to be taken into consideration in further studies. For *N. maculatus*, suggestive signs of gene flow between PNAC and Majorca were found that would warrant extended surveys in the region. For *N. lumbriciformis* from Vigo Estuary, microsatellite diversity was consistent with reported population data from northern Portugal (Monteiro et al. 2014, 2017). For *E. aequoreus* from PNIA Cytb diversity was also concordant with previous phylogeographic data of the species (Braga-Gonçalves et al. 2017). For *H. guttulatus* from PNIA, genetic diversity was consistent with previous studies in Galician coastal populations (López et al. 2015). This supports temporal stability and gene flow between PNIA and adjacent areas, associated with high habitat suitability for this species (Peiffer et al. 2024), and part of the South Atlantic lineage of the species (Riquet et al. 2019; Stacy et al. 2021). Population declines of seahorses in Mediterranean regions highlight the need for conservation efforts (Pierri et al. 2021).

Based on these results, we suggest that translocations of genetically distinct populations should be avoided, particularly between Atlantic and Mediterranean areas. Furthermore, we propose that protected areas be recognised as important genetic stocks for maintenance and connectivity with unprotected areas, as reported for threatened syngnathids (Planas et al. 2021; Stump et al. 2023). Therefore, long-term genetic studies could be carried out to monitor genetic diversity and whether populations maintain connectivity between protected and unprotected areas, to ensure the conservation of these species in the region, which, as umbrella taxa, may have positive impact on preserving coastal biodiversity (Monteiro et al. 2023).

### 4.3 | Conservation Applications

Within the context of threatened marine biodiversity, marine NPs are recognised by the IUCN as Category II protected areas, encompassing significant ecosystems and habitats while promoting education and recreation activities related to natural resources within a sustainable development framework. This study provides the first genetic assessment of syngnathid fishes in the Spanish marine NPs (PNIA and PNAC) using mitochondrial and microsatellite markers.

Genetic and morphological data revealed a limited diversity of syngnathid species differentially distributed between NPs, aiding in misclassification resolution and the identification of

cryptic lineages, such as a divergent Mediterranean population of *S. abaster* in PNAC. This information contributed to identify selected areas of occupation for different syngnathid species within the two Spanish marine NPs, associated to specific habitats and plant communities of conservation concern. It is crucial to promote the conservation of these areas, minimizing alterations of these ecosystems sensitive to navigational activities (e.g. recreation, transit, fishing). Additionally, temporary monitoring actions should be planned to validate incomplete data and detect changes resulting from habitat alteration and/or climate change.

In the period surveyed, genetic analyses revealed spatial and temporal stability of genetic diversity for dominant syngnathids in each NP, but differentiation between populations of the species detected in both Atlantic and Mediterranean NPs. Gene flow between NPs and nearby unprotected areas was detected, influencing demography and genetic diversity, which pinpoint the value of protected areas to preserve evolutionary processes for marine ecosystems and habitats, but also of connected adjacent areas which may benefit coordinated management and conservation actions. Monitoring total genetic diversity within and among populations at the intraspecific level will contribute to identify management units as a basis to support conservation actions.

Temporal evaluations of more abundant species in PNIA (*S. acus*) and PNAC (*S. typhle* and *S. abaster*) are recommended to explore long-term trends, as well as continuing to assess less abundant syngnathids, such as iconic seahorses. In this regard, citizen collaboration can contribute sighting records and/or indirect catches (through diving associations or artisanal fishermen) (Ruiz-Jarabo et al. 2024). In addition, genetic marker information could be useful to guide ex situ conservation activities in aquaria and captive breeding actions for endangered species. These efforts can also promote public education and awareness regarding marine biodiversity (Castejón-Silvo et al. 2023).

In conclusion, this study will contribute to advancing the biological knowledge of syngnathids genetic resources in PNIA and PNAC and connected adjacent areas outside of the NPs, as a basis to support the conservation of these umbrella species and the protection of valuable coastal habitats and ecosystems.

### Author Contributions

Conceptualisation: Carmen Bouza, Manuel Vera and Belén G. Pardo; field work: Miquel Planas and Inés Castejón-Silvo; formal analysis: Carmen Bouza, Manuel Vera and Belen G. Pardo; funding acquisition: Carmen Bouza and Miquel Planas; methodology: Carmen Bouza, Manuel Vera, Belén G. Pardo and Inés Castejón-Silvo; writing – original draft: Carmen Bouza; writing – review and editing: Manuel Vera, Belén G. Pardo, Miquel Planas, Inés Castejón-Silvo, Carmen Bouza. All authors have read and agreed to the published version of the manuscript.

### Acknowledgements

This project was funded by the Spanish Autonomous Agency of National Parks of the Ministry of Agriculture, Food and Environment (HIPPOPARQUES 1580S/2015). Thanks to the team members and collaborators of the coordinated project HIPPOPARQUES (1541S/2015), Xunta de Galicia and responsible personnel of Spanish National Parks for facilitating sampling permits. We are grateful for the support provided by J. Terrados, P. Arechavala and J. Castro from IMEDEA-CSIC, the Marine Research and Aquaculture Laboratory (CSIC) of the Government of the Balearic Islands (Directorate General for Fisheries and Marine Environment), the staff of the PNAC, and the Portixol and Cala Gamba nautical clubs. We would like to thank A. López for her contribution to previous studies on seahorses, as well as the collaboration and technical support of L. Bouzas, M. Villar, L. Insua and S. Gómez. Finally, we also sincerely appreciate the valuable comments and suggestions provided by Dr. Heidi Burdett and the anonymous reviewers, which have greatly contributed to improving the quality of this manuscript.

### Ethics and Permit Approval

Fish capture, handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (R.D. 1201/2005, 10th October) and the Regional Government Xunta de Galicia (Reference REGA ES360570202001/16/FUN/BIOL.AN/MPO02).

### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

All data (i.e. GenBank accession numbers of the identified haplotypes and microsatellite genotyping for each species) is provided in the Supplementary material.

### References

Allendorf, F. W., G. Luikart, and S. N. Aitken. 2013. *Conservation and the Genetics of Populations*. 2nd ed. Wiley-Blackwell Publishing Ltd.

Braga-Gonçalves, I., L. Cornetti, A. S. Couperus, C. J. G. Van Damme, and K. B. Mobley. 2017. "Phylogeography of the Snake Pipefish, *Entelurus Aequoreus* (Family: Syngnathidae) in the Northeastern Atlantic Ocean." *Biological Journal of the Linnean Society* 122: 787–800. https://doi.org/10.1093/biolinnean/blx112.

Casey, S. P., H. J. Hall, H. F. Stanley, and A. C. J. Vincent. 2004. "The Origin and Evolution of Seahorses (Genus *Hippocampus*): A Phylogenetic Study Using the Cytochrome b Gene of Mitochondrial DNA." *Molecular Phylogenetics and Evolution* 30: 261–272. https://doi.org/10.1016/j.ympev.2003.08.01.

Castejón-Silvo, I., J. Terrados, and B. Morales-Nin. 2023. "Citizen Science in the Study of Marine Biodiversity: The Case of Iconic and Cryptic Syngnathids." *Thalassas* 39: 679–686. https://doi.org/10.1007/s41208-023-00590-1.

CITES. 2024. "The Checklist of CITES Species Website." CITES Secretariat, Geneva, Switzerland. http://checklist.cites.org.

Diekmann, O. E., L. Gouveia, E. A. Serrão, and M. S. Van De Vliet. 2009. "Highly Polymorphic Microsatellite Markers for the Black Striped Pipefish, *Syngnathus Abaster*." *Molecular Ecology Resources* 9: 1460–1466. https://doi.org/10.1111/j.1755–0998.2009.02759.x.

Earl, D. A., and B. M. Vonholdt. 2012. "STRUCTURE HARVESTER: A Website and Program for Visualizing STRUCTURE Output and Implementing the Evanno Method." *Conservation Genetics Resources* 4: 359–361. https://doi.org/10.1007/s12686-011-9548-7.

Endo, T., M. Sekino, A. Fujiwara, and A. Sogabe. 2018. "Development and Characterization of 19 Novel Microsatellite Markers in the Pacific Seaweed Pipefish *Syngnathus Schlegeli* Using Next-Generation Sequencing." *Molecular Biology Reports* 45: 2831–2834. https://doi.org/10.1007/s11033-018-4396-0.

Evanno, G., S. Regnaut, and J. Goudet. 2005. "Detecting the Number of Clusters of Individuals Using the Software Structure: A Simulation

Study." *Molecular Ecology* 14: 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x.

Excoffier, L., and H. E. L. Lischer. 2010. "Arlequin Suite ver 3.5: A New Series of Programs to Perform Population Genetics Analyses Under Linux and Windows." *Molecular Ecology Resources* 10: 564–567. https://doi.org/10.1111/j.1755–0998.2010.02847.x.

Garcia, E., C. A. Rice, D. J. Eernisse, K. L. Forsgren, J. P. Quimbayo, and G. W. Rouse. 2019. "Systematic Relationships of Sympatric Pipefishes (*Syngnathus* spp.): A Mismatch Between Morphological and Molecular Variation." *Journal of Fish Biology* 95: 999–1012. https://doi.org/10.1111/jfb.14073.

Garner, B. A., S. Hoban, and G. Luikart. 2020. "IUCN red List and the Value of Integrating Genetics." *Conservation Genetics* 21: 795–801. https://doi.org/10.1007/s10592-020-01301-6.

Gilby, B. L., A. D. Olds, R. M. Connolly, et al. 2017. "Umbrellas can Work Under Water: Using Threatened Species as indicator and Management Surrogates Can Improve Coastal Conservation." *Estuarine, Coastal and Shelf Science* 199: 132–140. https://doi.org/10.1016/j.ecss.2017.10.003.

Goudet, J. 2001. "FSTAT, a Program to Estimate and Test Gene Diversities and Fixation Indices (Version 2.9.3)." http://www.unil.ch/izea/softwares/fstat.html.

Hablützel, P. I., and A. B. Wilson. 2011. "Notes on the Occurrence of *Syngnathus Rostellatus* (Teleostei: Syngnathidae) in the Mediterranean." *Marine Biodiversity Records* 4: e57. https://doi.org/10.1017/S1755267211000558.

Hall, T. A. 1999. "BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT." *Nucleic Acids Symposium Series* 41: 95–98.

Hamilton, H., N. Saarman, G. Short, et al. 2017. "Molecular Phylogeny and Patterns of Diversification in Syngnathid Fishes." *Molecular Phylogenetics and Evolution* 107: 388–403. https://doi.org/10.1016/j.ympev.2016.10.003.

Hübner, K., M. González-Wanguemert, O. E. Diekmann, and E. A. Serrão. 2013. "Genetic Evidence for Polygynandry in the Black-Striped Pipefish *Syngnathus Abaster*: A Microsatellite-Based Parentage Analysis." *Journal of Heredity* 104: 791–797. https://doi.org/10.1093/jhered/est049.

IUCN. 2024. "The IUCN red List of Threatened Species. Version 2023–1." http://www.iucnredlist.org.

Kopelman, N. M., J. Mayzel, M. Jakobsson, N. A. Rosenberg, and I. Mayrose. 2015. "Clumpak: A Program for Identifying Clustering Modes and Packaging Population Structure Inferences Across K." *Molecular Ecology Resources* 15: 1179–1191. https://doi.org/10.1111/1755–0998.12387.

Kuiter, R. H. 2009. "Seahorses and Their Relatives." Seaford: Aquatic Photographics, Australia.

Kumar, S., G. Stecher, and K. Tamura. 2016. "MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets." *Molecular Biology and Evolution* 33: 1870–1874. https://doi.org/10.1093/molbev/msw054.

Librado, P., and J. Rozas. 2009. "DnaSP v5: A Software for Comprehensive Analysis of DNA Polymorphism Data." *Bioinformatics* 25: 1451–1452. https://doi.org/10.1093/bioinformatics/btp187.

López, A., B. G. Pardo, M. Planas, P. Quintas, P. Martínez, and C. Bouza. 2012. "A Microsatellite Panel for Mating System Analysis and Broodstock Management of Captive Long-Snouted Seahorse *Hippocampus Guttulatus*." *Aquaculture* 356: 153–157. https://doi.org/10.1016/j.aquaculture.2012.05.021.

López, A., M. Vera, F. Otero-Ferrer, et al. 2010. "Species Identification and Genetic Structure of Threatened Seahorses in Gran Canaria Island (Spain) Using Mitochondrial and Microsatellite Markers." *Conservation Genetics* 11: 2431–2436. https://doi.org/10.1007/s10592-010-0116-6.

López, A., M. Vera, M. Planas, and C. Bouza. 2015. "Conservation Genetics of Threatened *Hippocampus Guttulatus* in Vulnerable Habitats in NW Spain: Temporal and Spatial Stability of Wild Populations With Flexible Polygamous Mating System in Captivity." *PLoS ONE* 10: e0117538. https://doi.org/10.1371/journal.pone.0117538.

Luo, W., H. Qu, J. Li, X. Wang, and Q. Lin. 2015. "A Novel Method for the Identification of Seahorses (Genus *Hippocampus*) Using Cross-Species Amplifiable Microsatellites." *Fisheries Research* 172: 318–324. https://doi.org/10.1016/j.fishres.2015.07.017.

Mkare, T. K., B. J. van Vuuren, and P. R. Teske. 2021. "Conservation Priorities in an Endangered Estuarine Seahorse Are Informed by Demographic History." *Scientific Reports* 11: 4205. https://doi.org/10.1038/s41598-021-83754-4.

Monteiro, N., S. Pinheiro, S. Magalhães, P. Tarroso, and A. Vincent. 2023. "Predicting the Impacts of Climate Change on the Distribution of European Syngnathids Over the Next Century." *Frontiers in Marine Science* 10: 1138657. https://doi.org/10.3389/fmars.2023.1138657.

Monteiro, N. M., D. Carneiro, A. Antunes, N. Queiroz, M. N. Vieira, and A. G. Jones. 2017. "The lek Mating System of the Worm Pipefish (*Nerophis Lumbriciformis*): A Molecular Maternity Analysis and Test of the Phenotype-Linked Fertility Hypothesis." *Molecular Ecology* 26: 1371–1385. https://doi.org/10.1111/mec.13931.

Monteiro, N. M., R. M. Silva, M. Cunha, A. Antunes, A. G. Jones, and M. N. Vieira. 2014. "Validating the Use of Colouration Patterns for Individual Recognition in the Worm Pipefish Using a Novel Set of Microsatellite Markers." *Molecular Ecology Resources* 14: 150–156. https://doi.org/10.1111/1755-0998.12151.

Montes, M. A., M. L. V. Cardoso, C. H. C. B. Neves, A. C. L. García, J. C. Da Silva, and R. B. Silveira. 2018. "Genetic Diversity and Populational Structure of the Seahorse *Hippocampus Reidi* (Syngnathidae) in North-Eastern Brazil: A Conservationist Approach." *Aquatic Conservation: Marine and Freshwater Ecosystems* 28: 1114–1122. https://doi.org/10.1002/aqc.2919.

Mwale, M., H. Kaiser, N. P. Barker, A. B. Wilson, and P. R. Teske. 2013. "Identification of a Uniquely Southern African Clade of Coastal Pipefishes *Syngnathus* spp." *Journal of Fish Biology* 82: 2045–2062. https://doi.org/10.1111/jfb.12130.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. "MICRO-CHECKER: Software for Identifying and Correcting Genotyping Errors in Microsatellite Data." *Molecular Ecology Notes* 4: 535–538. https://doi.org/10.1111/j.1471–8286.2004.00684.x.

OSPAR. 2008. "Liste OSPAR des espèces et Habitats menacés et/ou en déclin." 5 pp. https://www.ospar.org/work-areas/bdc/species-habitats.

Pääbo, S., W. K. Thomas, K. M. Whitfield, Y. Kumazawa, and A. C. Wilson. 1991. "Rearrangements of Mitochondrial Transfer RNA Genes in Marsupials." *Journal of Molecular Evolution* 33: 426–430.

Paetkau, P., R. Slade, M. Burden, and A. Estoup. 2004. "Genetic Assignment Methods for the Direct, Real-Time Estimation of Migration Rate: A Simulation-Based Exploration of Accuracy and Power." *Molecular Ecology* 13: 55–65. https://doi.org/10.1046/j.1365-294x.2004.02008.x.

Palumbi, S. R., A. Martin, S. Romano, W. O. McMillan, L. Stice, and G. Grabowski. 1991. "The Simple Fool's Guide to PCR, Ver. 2.0." Department of Zoology. University of Hawaii Special Publication, Hawaii.

Peiffer, F., A. R. Araujo Lima, S. Henriques, et al. 2024. "Habitat Suitability of Two Flagship Species, *Hippocampus* and *Hippocampus Guttulatus*, in the Atlantic Coast of the Iberian Peninsula—Implications for Conservation." *Global Ecology and Conservation* 53: e02993. https://doi.org/10.1016/j.gecco.2024.e02993.

Pierri, C., F. Cardone, G. Corriero, et al. 2021. "Density Decline in a Mediterranean Seahorse Population: Natural Fluctuations or New Emerging Threats?" *Frontiers in Marine Science* 8: 692068. https://doi.org/10.3389/fmars.2021.692068.

Piry, S., A. Alapetite, J. M. Cornuet, D. Paetkau, L. Baudouin, and A. Estoup. 2004. "GENECLASS2: A Software for Genetic Assignment and First-Generation Migrant Detection." *Journal of Heredity* 95: 536–539. https://doi.org/10.1093/jhered/esh074.

Planas, M. 2022. "Ecological Traits and Trophic Plasticity in the Greater Pipefish *Syngnathus Acus* in the NW Iberian Peninsula." *Biology* 11, no. 5: 712. https://doi.org/10.3390/biology11050712.

Planas, M., C. Piñeiro-Corbeira, C. Bouza, et al. 2021. "A Multidisciplinary Approach to Identify Priority Areas for the Monitoring of a Vulnerable Family of Fishes in Spanish Marine National Parks." *BMC Ecology and Evolution* 21: 4. https://doi.org/10.1186/s12862-020-01743-z.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. "Inference of Population Structure Using Multilocus Genotype Data." *Genetics* 155, no. 2: 945–959. https://doi.org/10.1093/genetics/155.2.945.

Rannala, R., and J. L. Mountain. 1997. "Detecting Immigration by Using Multilocus Genotypes." *Proceedings of the National Academy of Sciences* 94: 9197–9201. https://doi.org/10.1073/pnas.94.17.9197.

Riquet, F., C. Liautard-Haag, L. Woodall, et al. 2019. "Parallel Pattern of Differentiation at a Genomic Island Shared Between Clinal and Mosaic Hybrid Zones in a Complex of Cryptic Seahorse Lineages." *Evolution* 73: 817–835. https://doi.org/10.1111/evo.13696.

Roth, O., I. Keller, S. H. Landis, W. Salzburger, and T. B. H. Reusch. 2012. "Hosts Are Ahead in a Marine Host-Parasite Coevolutionary Arms Race: Innate Immune System Adaptation in Pipefish *Syngnathus Typhle* Against *Vibrio* Phylotypes." *Evolution* 66: 2528–2539. https://doi.org/10.1111/j.1558–5646.2012.01614.x.

Roth, O., M. H. Solbakken, O. K. Torresen, et al. 2020. "Evolution of Male Pregnancy Associated With Remodeling of Canonical Vertebrate Immunity in Seahorses and Pipefishes." *Proceedings of the National Academy of Sciences of the United States of America* 117: 9431–9439. https://doi.org/10.1073/pnas.1916251117.

Rousset, F. 2008. "GENEPOP'007: A Complete Re-Implementation of the GENEPOP Software for Windows and Linux." *Molecular Ecology Resources* 8: 103–106. https://doi.org/10.1111/j.1471–8286.2007.01931.x.

Ruiz-Jarabo, I., J. Hernández-Urcera, S. Pereira, I. Sobrino, J. A. López, and M. Planas. 2024. "Occurrence of Seahorses *Hippocampus* spp. in the Southernmost Part of Western Europe: A New Maximum Depth Record." *Animals* 14, no. 16: 2328. https://doi.org/10.3390/ani14162328.

Sanna, D., F. Biagi, H. B. Alaya, et al. 2013. "Mitochondrial DNA Variability of the Pipefish *Syngnathus Abaster*." *Journal of Fish Biology* 82: 856–876. https://doi.org/10.1111/jfb.12027.

Sogabe, A., and M. Takagi. 2013. "Population Genetic Structure of the Messmate Pipefish *Corythoichthys Haematopterus* in the Northwest Pacific: Evidence for a Cryptic Species." *Springerplus* 2: 408. https://doi.org/10.1186/2193-1801-2-408.

Stacy, R., J. Palma, M. Correia, A. B. Wilson, J. P. Andrade, and R. Castilho. 2021. "The Paradox of Retained Genetic Diversity of *Hippocampus Guttulatus* in the Face of Demographic Decline." *Scientific Reports* 11: 10434. https://doi.org/10.1038/s41598-021-89708-0.

Stolting, K. N., and A. B. Wilson. 2007. "Male Pregnancy in Seahorses and Pipefish: Beyond the Mammalian Model." *BioEssays* 29: 884–896. https://doi.org/10.1002/bies.20626.

Stump, E., J. S. Rosenfeld, and A. C. J. Vincent. 2023. "Habitat Associations and Threat Vulnerabilities of Seahorses and Pipefishes (Syngnathidae) in Biscayne National Park, Florida, USA." *Bulletin of Marine Science* 99: 441–466. https://doi.org/10.5343/bms.2022.0067.

Valladares, S., R. Bañón, A. López, et al. 2014. "First Records of the Seahorse *Hippocampus* in Galician Waters (NW Spain)." *Cybium* 38: 74–76.

Vincent, A. C. J., S. J. Foster, and H. J. Koldewey. 2011. "Conservation and Management of Seahorses and Other Syngnathidae." *Journal of Fish Biology* 78: 1681–1724. https://doi.org/10.1111/j.1095–8649.2011.03003.x.

Waycott, M., C. M. Duarte, T. J. B. Carruthers, et al. 2009. "Accelerating Loss of Seagrasses Across the Globe Threatens Coastal Ecosystems." *Proceedings of the National Academy of Sciences of the United States of America* 106: 12377–12381. https://doi.org/10.1073/pnas.0905620106.

Weiss, S.-E., A. Emami-Khoyi, H. Kaiser, et al. 2022. "The Last two Remaining Populations of the Critically Endangered Estuarine Pipefish Are Inbred and Not Genetically Distinct." *Frontiers in Marine Science* 8: 756595. https://doi.org/10.3389/fmars.2021.756595.

Wilson, A. B., J. Ashe, M. Padron, and H. Hamilton. 2021. "Comprehensive Genus-Wide Screening of Seahorse Microsatellite Loci Identifies Priority Species for Conservation Assessment." *Conservation Genetics Resources* 13: 221–230. https://doi.org/10.1007/s12686-021-01198-4.

Wilson, A. B., and I. E. Veraguth. 2010. "The Impact of Pleistocene Glaciation Across the Range of a Widespread European Coastal Species." *Molecular Ecology* 19: 4535–4553. https://doi.org/10.1111/j.1365-294X.2010.04811.x.

Wilson, A. B., A. Vincent, I. Ahnesjö, and A. Meyer. 2001. "Male Pregnancy in Seahorses and Pipefishes (Family Syngnathidae): Rapid Diversification of Paternal Brood Pouch Morphology Inferred From a Molecular Phylogeny." *Journal of Heredity* 92: 159–166. https://doi.org/10.1093/jhered/92.2.159.

Woodall, L. C., H. J. Koldewey, J. T. Boehm, and P. W. Shaw. 2015. "Past and Present Drivers of Population Structure in a Small Coastal Fish, the European Long Snouted Seahorse *Hippocampus Guttulatus*." *Conservation Genetics* 16: 1139–1153. https://doi.org/10.1007/s10592-015-0728-y.

Woodall, L. C., F. Otero-Ferrer, M. Correia, et al. 2018. "A Synthesis of European Seahorse Taxonomy, Population Structure, and Habitat Use as a Basis for Assessment, Monitoring and Conservation." *Marine Biology* 165: 19. https://doi.org/10.1007/s00227-017-3274-y.

## Supporting Information

Additional supporting information can be found online in the Supporting Information section.