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AQUATIC CRYPTIC DIVERSITY

A North–South genetic divide in marine Gastrotricha: distinct Mediterranean lineages across four species

Agata Cesaretti · Jan-Niklas Macher · M. Antonio Todaro

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Abstract The aim of this study is to test whether four marine gastrotrich species shared between the North Sea and the Mediterranean Sea represent single, pan-European taxa or instead comprise hidden, genetically structured lineages. Using single-specimen whole-genome sequencing, multigene phylogenetics (18S, 28S, COI), and multiple species-delimitation methods, we analyzed populations of *Xenotrichula intermedia*, *Halichaetonotus aculifer*, *H. atlanticus*, and *Turbanella bocqueti* sampled from the North Sea and the Mediterranean Sea. All four investigated species were resolved into at least two phylogenetic lineages, consistently separating North Sea from Mediterranean populations. In *X. intermedia*, Mediterranean specimens further split into distinct Ligurian and Adriatic lineages, revealing deep cryptic diversity despite morphological uniformity. Distance-based and coalescent-based delimitation approaches strongly corroborated these divisions, supporting the existence of pronounced genetic divergence and geographic structuring across the investigated populations. Additional internal structuring was detected within northern populations of both *H. atlanticus* and *T. bocqueti*, with *H. atlanticus* showing a shallow but still detectable North Sea–Mediterranean split. Overall, our results demonstrate a consistent genetic differentiation between North Sea and Mediterranean populations and uncover substantial hidden diversity in the European marine gastrotrichs of the examined areas, challenging assumptions of broad dispersal and highlighting the need for integrative taxonomic reassessment of meiofaunal biogeography.

Keywords Gastrotrich · Marine meiofauna · Phylogeography · Cryptic diversity · Mediterranean fauna · Meiofauna paradox

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A. Cesaretti (✉) · M. A. Todaro
Department of Life Sciences, University of Modena and Reggio Emilia, Via Giuseppe Campi, 213/D, 41125 Modena, Italy
e-mail: agata.cesaretti@unimore.it

J.-N. Macher
Naturalis Biodiversity Center, Leiden, The Netherlands

J.-N. Macher
Department of Environmental Biology, Institute of Environmental Sciences (CML), Leiden University, Leiden, The Netherlands

M. A. Todaro
National Biodiversity Future Center (NBFC), Palermo, Italy

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Introduction

Meiobenthic metazoans pose a long-standing biogeographic paradox: despite their limited dispersal abilities, they are often reported to have remarkably broad geographic ranges. This pattern has traditionally been interpreted through the "everything is everywhere, but the environment selects" (EiE) hypothesis (Baas Becking, 1934; Sterrer, 1973; Artois et al., 2011; Fontaneto & Brodie, 2011). However, meiofaunal organisms typically have short life cycles, produce few offspring, lack pelagic larvae, and remain closely associated with benthic substrates, traits that should constrain long-distance dispersal (Giere, 2009; Leasi & Todaro, 2009).

This apparent contradiction is particularly evident in Gastrotricha, a phylum of microscopic, free-living aquatic invertebrates comprising more than 900 described species across the orders Macrodasyida and Chaetonotida (Todaro et al., 2019; Saponi & Todaro, 2024; Cesaretti et al., 2024; Souid et al., 2024, 2026; Minowa et al., 2025; Saponi et al., 2026). Gastrotrichs are typically benthic, interstitial organisms that glide using ventral cilia and possess structural adaptations such as a flexible, often ornamented cuticle and a duo-gland adhesive system (Rieger & Rieger, 1977; Tyler & Rieger, 1980). They are primarily hermaphroditic, with internal fertilization, although parthenogenesis is widespread in some groups; development is direct and no planktonic dispersal stage is known (Hummon & Hummon, 1992; Gammuto et al., 2024; Kosakyan et al., 2026; Wallnoefer et al., 2026).

Many marine gastrotrichs have been reported from widely separated regions and even considered cosmopolitan (Ruppert, 1977; Todaro et al., 1996; Hummon, 2010; Chatterjee & Artois et al., 2011; Todaro, 2021). However, such distributions are increasingly recognized as artifacts of underestimated diversity, often masking complexes of cryptic or pseudocryptic species (Giere, 2009; Leasi & Todaro, 2009; Fontaneto & Brodie, 2011; Kånneby et al., 2012; Kieneke et al., 2012; Meyer-Wachsmuth et al., 2014; Cerca et al., 2018; Magpali et al., 2021). This underestimation is further compounded by methodological limitations and historical inconsistencies in taxonomic practice, including low-resolution microscopy, lack of standardized descriptions, and incomplete voucher documentation (Leasi & Todaro, 2009; Artois et al., 2011; Cerca et al., 2018; Chatterjee & Todaro, 2021). In addition, global distribution patterns remain incompletely resolved due to uneven sampling effort and persistent taxonomic impediments (Todaro et al., 2009; Todaro et al., 2025); Garraffoni & Balsamo, 2017; Chatterjee et al., 2019; Garraffoni et al., 2021, 2024).

Within Europe, the North Sea and the Mediterranean Sea represent two contrasting marine systems connected by a continuous Atlantic coastline that may facilitate dispersal of coastal meiofauna (Sterrer, 1973; Artois et al., 2011). The North Sea is a relatively open and well-mixed basin, whereas the Mediterranean Sea is a semi-enclosed system with a complex geological and oceanographic history. The Messinian Salinity Crisis (~5.96–5.33 Ma) led to extensive marine extinctions, followed by recolonization from Atlantic lineages after the reopening of the Strait of Gibraltar (Bianchi & Morri, 2000; Blanc, 2002; van Dijk et al., 2023). Today, the Mediterranean hosts more than 17,000 marine species and shows an average endemism rate of approximately 20% (Bianchi & Morri, 2000; Coll et al., 2010). Its subdivision into multiple basins and complex circulation patterns (Pinardi & Masetti, 2000) may promote population differentiation. Despite this, the gastrotrich fauna of the Mediterranean shows substantial overlap with that reported from northern Europe (Hummon, 2010; Garraffoni and Balsamo, 2017), raising questions about connectivity and true diversity.

Within this framework, species reported from both northern Europe and the Mediterranean Sea provide particularly useful systems for testing whether apparently broad distributions reflect real connectivity or conceal geographically structured, cryptic diversity. More specifically, the aim of the present study was to determine whether the apparently broad European distributions of four marine gastrotrich species reflect true pan-European taxa or instead conceal geographically structured evolutionary lineages that may correspond to cryptic species.

In the present study, we focus on four marine gastrotrich species shared between these two regions: *Xenotrichula intermedia* Remane, (1934), *Halichaetonotus aculifer* (Gerlach, 1953), *Halichaetonotus atlanticus* Kisielewski, (1988), and *Turbanella bocqueti* Kaplan, (1958) (Fig. 1). The examined taxa

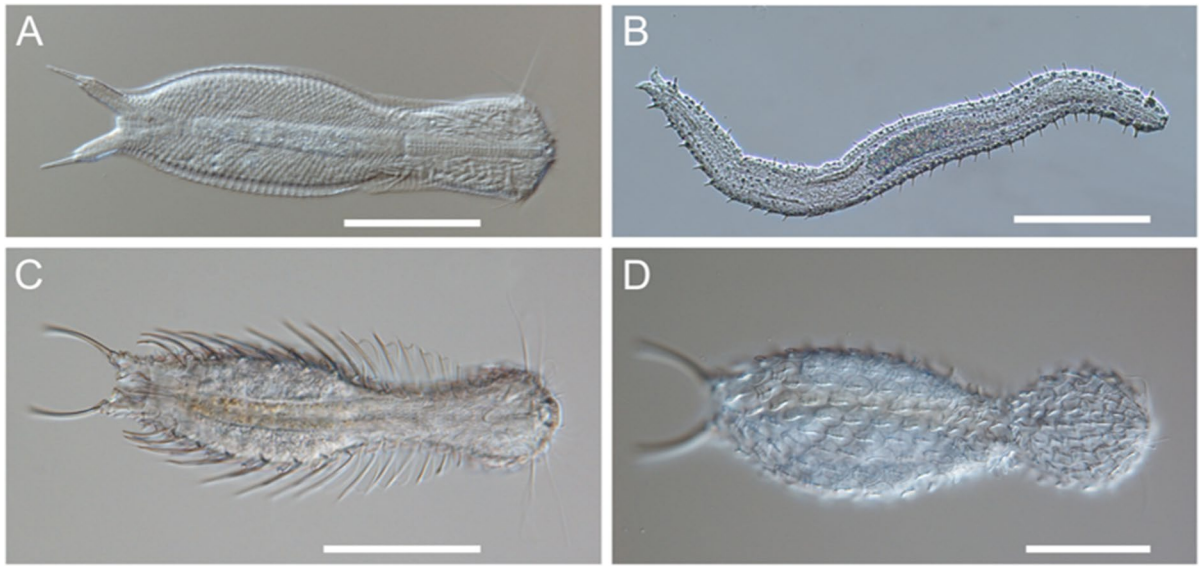


Fig. 1 Micrographs of the species studied in the present work. **A** *Xenotrichula intermedia* Remane, (1934). **B** *Turbanella bocqueti* Kaplan, (1958). **C** *Halichaetonotus aculifer* (Gerlach, 1953). **D** *Halichaetonotus atlanticus* Kisielewski, (1988). Scale bars: A, C, D = 50 µm; B = 250 µm. Differential interference contrast microscopy (Nomarski)

include representatives of both gastrotrich orders, Chaetonotida (*X. intermedia*, *H. aculifer*, *H. atlanticus*) and Macrodasyida (*T. bocqueti*), and encompass the two reproductive strategies known in the phylum, namely hermaphroditism with internal cross-fertilization (*X. intermedia*, *T. bocqueti*) and parthenogenesis (*H. aculifer*, *H. atlanticus*). The selected species also encompass different ecological strategies, ranging from predominantly littoral taxa (*X. intermedia* and the two *Halichaetonotus* species) to the mainly sublittoral *Turbanella bocqueti*, while differing in traits that may influence their dispersal potential (Ruppert, 1977).

Xenotrichula intermedia represents an especially suitable subject for investigating cryptic diversity and geographic structuring in marine meiofauna. This minute (200 µm) species is cosmopolitan: originally described from northern Europe, it has since been reported from a wide range of regions, including the Mediterranean Sea, the Atlantic Ocean, India, and Somalia (Ruppert, 1979; Todaro et al., 1996; Hummon, 2010). In Europe, it is widely distributed along the northern and western coasts, from the Baltic Sea to the North Sea and the British Isles, extending southward to Portugal (Remane, 1934; Hummon, 2008, 2010). In the Mediterranean Sea, it was first recorded in Tuscany (Gerlach, 1953) and has since been reported along most Italian coastlines, as well as in Algeria, Tunisia, Egypt, and Greece (Todaro et al., 2001; Hummon, 2010). Previous studies have shown high genetic variability among geographically distant populations of *X. intermedia* (Todaro et al., 1996) and revealed hidden morphological divergence detectable only through confocal microscopy (Leasi & Todaro, 2009), supporting the existence of cryptic or pseudocryptic species. Additional lineages have been identified in other regions, including the Persian Gulf and the western Atlantic (Hochberg & Litvaitis, 2003). More recently, Munter & Kieneke (2017) proposed the existence of a pan-European lineage of *X. intermedia* based on similarities in muscular architecture among North Sea, Baltic, and Mediterranean populations. However, this hypothesis has not yet been tested using molecular data, leaving the extent of connectivity between these regions unresolved. Similar uncertainty also applies to other gastrotrich species reported from both northern Europe and the Mediterranean, including those investigated in this study, whose apparently broad distributions may likewise reflect either ongoing connectivity or overlooked lineage diversity.

Halichaetonotus aculifer is a small (170 µm) chaetonotidan species, originally described from Tuscany (Gerlach, 1953) and now known to be

widespread throughout the Mediterranean basin (Hummon, 2010). In Europe, it occurs commonly along the Baltic Sea, the North Sea and Atlantic coasts, from Poland to France (Hummon, 2010; Kolicka, 2015). The species has also been repeatedly recorded along the Atlantic and Gulf coasts of the United States (Todaro et al., 1995; Hummon, 2010).

Halichaetonotus atlanticus is far less frequently encountered than its congener. Described from the Atlantic coast of France (Kisielewski, 1988), all subsequent records of this species come exclusively from the Mediterranean Sea, specifically from a few sites along the Tyrrhenian coast of Italy (Todaro et al., 2001). The rarity of *H. atlanticus* is notable given its shared reproductive mode and overall morphological similarity to *H. aculifer*. Moreover, both examined *Halichaetonotus* species inhabit littoral and sublittoral sediments (Todaro et al., 2001). *H. atlanticus* is unmistakably distinguished by the presence of noticeable, characteristic transverse ribs on the keel and scale surfaces (Kisielewski, 1988).

Finally, *Turbanella bocqueti* is a relatively large macrodasyidan, reaching up to 1320 µm in length (Hummon, 2008). First described from Normandy, France, it is common along the European Atlantic coast and in the British Isles and has been recorded also from the German island of Helgoland (Kaplan, 1958; Hummon, 2010). In the Mediterranean Sea, it was first recorded in Tuscany (Tongiorgi, 1975) and is now known from several Italian regions, Greece, and North Africa (Todaro et al., 2001). Outside of Europe, *T. bocqueti* has been reported from the Gulf of Mexico (Felder & Camp, 2009). The species typically inhabits fine sands in littoral and sublittoral zones and is remarkable for its ability to colonize anaerobic or poorly oxygenated sediments, where it behaves as a facultative thiobiont (Boaden, 1974; Tongiorgi, 1975; Hummon, 2008).

These four species were selected because they encompass a broad range of taxonomic, reproductive, ecological, and dispersal characteristics within marine Gastrotricha, while also being the only taxa for which sufficiently sampled populations from both the North Sea and the Mediterranean Sea were available for comparative molecular analyses. Using this comparative approach, we generated multilocus molecular datasets from single specimens by means of next-generation sequencing and analyzed them through multigene phylogenetic reconstruction and complementary species-delimitation approaches. The contrasting taxonomic and biological characteristics of the selected species also allowed us to assess whether the occurrence of cryptic species is species-specific or instead represents a broader and potentially pervasive feature among European marine gastrotrichs.

Materials and methods

Sampling and identification

North Sea and Mediterranean populations of the four focal species described above were analyzed. The North Sea specimens were collected in the Netherlands during a workshop on local meiofauna (Macher et al., 2024; Macher & Creuwels, 2024), whereas the Mediterranean specimens were collected along the Italian coastline as part of ongoing research on the national marine gastrotrich fauna (e.g., Saponi et al., 2024; Todaro & Rebecchi, 2025) (Fig. 2). In all cases, shortly after collection, the gastrotrichs were extracted from the sandy substrate using a 7% $MgCl_2$ narcotizing solution (Todaro et al., 2019). Specimens were then studied under a Nomarski microscope (Leitz Dialux 20 or Nikon Eclipse 90i), and high-resolution photomicrographs were taken as vouchers using a Nikon DS Fi3 digital camera controlled through the Nikon NIS Elements D software (v. 4.6). All specimens (see Fig. 1) were identified based on comparison with the diagnostic metric and meristic characters reported in the original descriptions and subsequent taxonomic literature. The four species investigated in this study have all been repeatedly reported from the sampled Mediterranean and North Sea regions by several authors, and their diagnostic morphological characteristics are well documented in the available taxonomic literature (cited in the introduction).

For each species at each sampling locality, one or a few representative specimens were examined in detail to establish the species identity based on the available taxonomic literature. Once the species identity had been established from these representative specimens, additional individuals displaying the same main diagnostic



Fig. 2 Map detailing the sampling sites of specimens involved in the study. Specimen vouchers and population names correspond to those reported in Table S1. Populations are color-coded according to the geographical region (Northern or Southern Europe)

character combination were assigned to the corresponding species by inference. This strategy is commonly adopted in population genetic studies of microscopic meiofaunal organisms and closely follows the approach used by Kieneke et al. (2012) in their population genetic study of the genus *Turbanella*. Complete morphometric characterization of all sequenced specimens was not possible, because detailed morphological examination of gastrotrichs typically requires prolonged microscopic observation and extensive compression of the specimen, a procedure that often results in its destruction and therefore precludes subsequent DNA extraction.

The identified specimens were preserved in 95% ethanol at −20 °C in 2-mL Eppendorf tubes until further processing. A complete list of sequenced specimens, together with GenBank accession numbers, names of the sampling locations and their geographic coordinates, is provided in Supplementary Table S1. No special permissions or permits were required to collect these organisms, as gastrotrichs

are microscopic, non-pathogenic, and not subject to collection restrictions. The fieldwork did not involve any endangered or protected species, and all sampling was conducted in publicly accessible areas.

DNA extraction, amplification and sequencing

Whole-genome amplification followed by Oxford Nanopore sequencing was adopted to recover multiple molecular markers from single gastrotrich specimens, from which only very small amounts of genomic DNA can be obtained. Single ethanol fixed specimens were rinsed in clean absolute ethanol and transferred each into a sterile 0.5-ml microtube using a clean, dedicated glass micro-pipette. The tubes with the specimens were left overnight at 25 °C in a cleaned Memmert UN 30 lab oven to eliminate any residual ethanol through evaporation. The DNA extraction and whole-genome amplification (WGA) steps were carried out in a UV-sterilized hood using the REPLI-g Single Cell Kit (QIAGEN®), following the manufacturer's protocol. Before sequencing, the amplified genomic DNA was cleaned with AMPure XP beads and then processed for endonuclease digestion using a T7 Endonuclease digestion kit, following the protocol of Lee et al. (2023). This step was included because, during the optimization of our sequencing protocol, we observed that untreated WGA products, consisting of long, branched DNA molecules, could clog the nanopores, thereby reducing sequencing efficiency (resulting estimated bases 4.73 Gb, reads = 1.13 M; see for comparison Supplementary Table S1). Next, the product was processed to create a sequencing library using the Native Barcoding Kit 24V14, following the manufacturer's protocol. The libraries ran on a PromethION 2 Solo NanoPore sequencing device (Oxford Nanopore Technologies).

Gene mining

The obtained WGS data were analyzed through a bioinformatic pipeline, optimized from Kumar et al. 2013 (see also Serra et al., 2020; Gammuto et al., 2024; Saponi et al. 2024). The quality of the reads was evaluated through the FASTQC software (Andrews, 2010), and the reads matching ribosomal and mitochondrial genes were identified through Blastn and tBlastn analysis. As queries, we used published gastrotrich sequences obtained from the public NCBI GenBank database. The software tools StructRNAfinder (https://structrnafinder.integrativebioinformatics.me/) (Arias-Carrasco et al., 2018) and MITOs (http://mitos.bioinf.uni-leipzig.de/help.py) (Bernt et al., 2013) were used to examine the reads of interest isolated from the sequenced data and to confirm the location of the identified genes. For each gene sequence, multiple independent reads were compared to create a final consensus sequence and minimize the likelihood of sequencing errors. The reads contributing to each consensus sequence showed a high degree of sequence congruence, with little or no detectable intraindividual variation. BioEdit v.7.2.5 Freeware (Hall, 1999) and MEGA X (Kumar et al., 2018) were used to manually align, examine, and, when necessary, assemble the extracted sequences. Once again, publicly available data obtained from GenBank were used as a reference to verify the reliability of the methodology and the validity of the results.

Gene alignment and phylogenetic analysis

For consistency, the multilocus phylogenetic analysis was based exclusively on molecular markers newly generated in the present study. This ensured that all specimens included in the concatenated dataset were represented by sequences obtained using the same sequencing strategy and characterized by comparable marker coverage and sequence length. The sequences of each of the three genes employed in the study (18S, 28S, COI) were aligned separately. The alignments were carried out using the MUSCLE (multiple sequences comparison by log expectation) algorithm (Edgar, 2004) as implemented in the MEGA X software package. The mitochondrial protein-coding cytochrome c oxidase subunit I (COI) sequences were aligned using the invertebrate mitochondrial genetic code. Before codon alignment, the COI sequences were individually examined for their correct reading frame using the MEGA X software. The aligned datasets were trimmed to match the length of the majority of taxa. The resulting curated datasets comprised 1866 bp (18S rDNA), 4233 bp (28S rDNA), and 1520 bp (COI), while the final concatenated matrix included 7619 nucleotide positions.

The phylogenetic analysis on the concatenated dataset was conducted using the Bayesian inference (BI) approach, on the program MrBayes v.3.2.7 (Ronquist et al., 2012). Model selection was performed independently for each gene partition using the Model Finder tool implemented in MEGA X, based on the corrected Akaike information criterion (AIC) and the Bayesian information criterion (BIC). The best-fitting models were K2P+FQ+I for the 18S and 28S partitions and HKY+F+I for the COI partition. Because these exact model formulations are not all implemented in MrBayes, the more general and parameter-rich GTR+G+I model was applied to all three partitions in the Bayesian phylogenetic analyses. This model has been widely employed in previous multilocus phylogenetic studies of Gastrotricha and was therefore considered an appropriate choice for the present analyses (e.g., Todaro et al., 2011; Kånneby et al., 2012).

The BI tree search on the final concatenated dataset ran with two parallel runs, on eight independent Markov chains performed in 6,000,000 generations, sampling the trees every 100 generations and with a relative burn-in fraction of 25%. Convergence of the Markov chain Monte Carlo (MCMC) analyses was confirmed with the in-built diagnostics of the program: the average standard deviation of split frequencies approached zero, the potential scale reduction factor (PSRF) converged to 1.00 for all parameters, and the effective sample sizes (ESS) of all parameters were >200. The resulting tree was visualized using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/ software/figtree/). The tree was computed as unrooted and then rooted for visualization on *Turbanella bocqueti*. This taxon was chosen as the outgroup because it is the only macrodasyidan species included in this study. To improve reading clarity, the final tree was edited using CorelDraw X7 (Corel Corporation, Ottawa, Canada).

DNA species delimitation

Species delimitation was performed through the analysis of mitochondrial COI gene sequences. This marker was selected because of its faster evolutionary rate compared with the nuclear ribosomal markers and its widespread use in species-delimitation studies on metazoans, including the limited number of comparable studies available for Gastrotricha (Gissi et al., 2008; Kieneke et al., 2012; Kånneby et al., 2012; Rataj Križanová and Vďačný, 2023).

The multilocus dataset was used to establish the primary phylogenetic framework, whereas the more inclusive COI dataset was subsequently employed for the species-delimitation analyses, allowing the incorporation of all comparable published sequences and thereby maximizing the available molecular evidence for the investigated taxa.

Of the eight potentially useful COI sequences identified in the NCBI GenBank nucleotide database, five showed substantial overlap with the regions obtained through our sequencing. Four belong to *Turbanella bocqueti*: two are derived from specimens collected in northern Europe (France, Normandy, near the English Channel; Ireland) and two from the Mediterranean coast of France (Todaro et al., 2011; Kieneke et al., 2012). The fifth sequence belongs to a *Xenotrichula intermedia* specimen collected from the coast of Tunisia (Todaro et al., 2011). These sequences were included in the analyses for their respective taxa. We also included in the analyses four COI sequences of *Halichaetonotus atlanticus* (Ht758, Ht773, Ht834, and Ht796) from the Netherlands, Naturalis Biodiversity Center in Leiden (for details see Macher et al., 2024).

Molecular species boundaries were inferred by separately analyzing the COI sequence dataset of each species using a suite of methods: (i) 4× rule (Birky et al. 2010); (ii) ABGD (Automatic Barcode Gap Discovery) (Puillandre et al., 2012); (iii) ASAP (Assemble Species by Automatic Partitioning) tests (Puillandre et al., 2021); (iv) GMYC (General Mixed Yule Coalescent) model (Pons et al., 2006); v) PTP (Poisson Tree Processes) and vi) bPTP (Bayesian Poisson Tree Processes) model (Zhang et al., 2013). Prior to the analysis, the COI sequences were aligned and trimmed to the shortest length resulting in 549 bp for *X. intermedia*, 679 bp for *Halichaetonotus aculifer*, 657 bp for *H. atlanticus* and 646 bp for *Turbanella bocqueti*.

Under the model of the 4× rule, clusters of sequences in which ingroup variation is at least fourfold lower than the divergence between clusters are inferred to represent distinct species (Birky et al., 2010). This single-locus approach assumes neutral evolution of the marker, absence of recombination, and negligible gene flow between lineages, as well as approximate population size stability. Calculations of the K (the average pair

distance between clusters) over Theta (estimation of the ingroup genetic diversity) rates were performed with the "KoT—K over Theta" software, ver. 1 (Spori et al., 2021), setting the KoT threshold value first at 4 and then at 6, in order to reduce the possibility of oversplitting.

The ABGD and ASAP tests were conducted on the SpartExplorer web platform (https://spartexplorer.mnhn.fr/) (Miralles et al., 2022). Both methods are single-locus species-delimitation approaches based on genetic distances. ABGD employs a distance threshold estimated from the intraspecific diversity and the interspecific diversity. ASAP implements a hierarchical clustering algorithm that only uses pairwise genetic distances. Both tools ran with the default options, applying the Jukes–Cantor substitution model, on the COI alignments of each species. The uncorrected pairwise distances (p-distances) were also calculated and examined using the Compute Pairwise Distances function in MEGA X.

Ultrametric trees for the GMYC analyses were inferred using BEAST v2.6.7 (Bouckaert et al., 2019). Run parameters were configured in BEAUti v2.6.7 to generate the corresponding XML input files. No temporal calibration (fossil, geological, or secondary calibration) was applied because no reliable fossil record or secondary calibration points are currently available for Gastrotricha. Consequently, the resulting ultrametric trees were interpreted exclusively within the framework of an uncalibrated relative species-delimitation analysis. All analyses were conducted under a strict molecular clock model and a Yule tree prior, following the original formulation of the GMYC method (Pons et al., 2006). Under these conditions, the objective was not to estimate divergence times, but to obtain ultrametric trees suitable for identifying the transition between interspecific diversification and intraspecific coalescent processes. The GMYC model test was then performed using the R package *splits* (Fujisawa & Barraclough, 2013).

The PTP analyses were conducted in the Exelixis Lab species delimitation web server (https://species.h-its.org/ptp/) (Zhang et al., 2013), using the COI dataset. Settings were kept as default, following the indications for small trees (100,000 MCMC generations, thinning set at 100, burn-in set at 0.1). Sequences from *Xenotrichula* sp. 1 (GenBank AN: JN185545) and *Turbanella hyalina* (GenBank AN: JN099957) were used as outgroups in the tree-based analyses, respectively, for the *X. intermedia* and *T. bocqueti* datasets.

The genetic relationships between the specimens at the population level were visualized through haplotype network analyses, conducted with the TCS model in PopART v. 1.7 (Leigh and Bryant, 2015). The haplotype files used as input, including information about haplotype identity, origin and frequencies, were obtained with the DnaSP software v. 6.12.03 (Rozas et al., 2017). To improve reading clarity, the resulting networks were edited using CorelDraw X7 (Corel Corporation, Ottawa, Canada).

Results

Sequencing

In the present study, we obtained a total of 141 new gene sequences, belonging, respectively, to 15 *X. intermedia* specimens (10 Mediterranean, 5 from the North Sea), 10 *H. aculifer* specimens (5 Mediterranean, 5 from the North Sea), 9 T. *bocqueti* specimens (5 Mediterranean, 4 from the North Sea), and 8 *H. atlanticus* specimens (5 Mediterranean, 3 from the North Sea). Oxford Nanopore whole-genome sequencing produced data with an average Q score of 18–19. Detailed sequencing statistics, including the total number of reads, mean read length and base coverage for each specimen, are reported in Supplementary File S1.

Phylogenetic analysis

The Bayesian phylogenetic analysis conducted on the concatenated dataset (18S + 28S + COI) produced a well-resolved topology, with most relevant nodes showing posterior probability values between 0.98 and 1.00. The overall topology is fully consistent with the current taxonomic classification of the examined species, with each species recovered as a distinct and well-supported clade, including the two *Halichaetonotus* taxa.

The analysis further resolved all four examined species into at least two distinct phylogenetic lineages, consistently separating North Sea and Mediterranean populations, generally with strong statistical support. The lowest support value was recovered for the Mediterranean *T. bocqueti* lineage (Tbo_Med), which was only moderately supported (pp = 0.91) (Fig. 3).

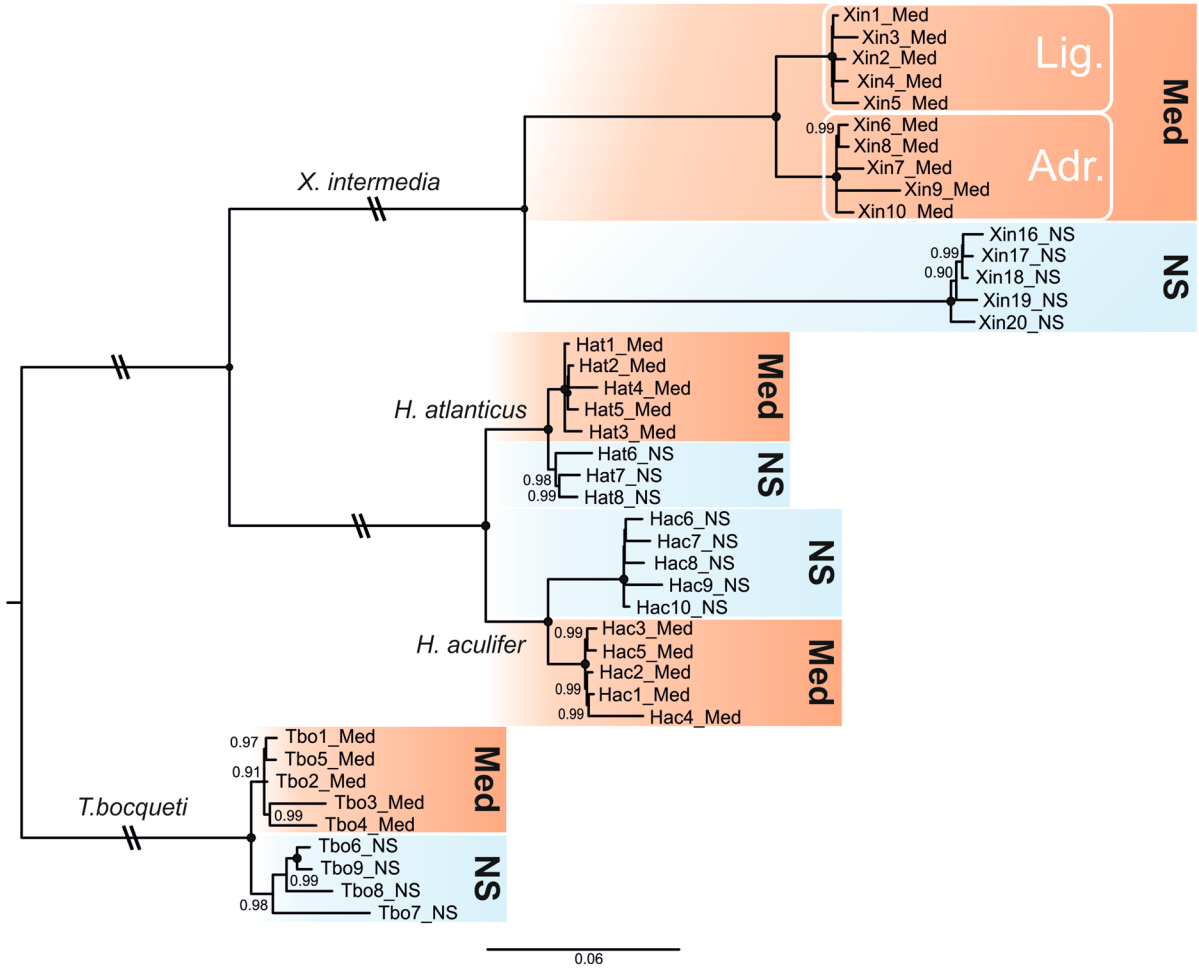


Fig. 3 Phylogenetic relationships of the examined species inferred from Bayesian analysis of the concatenated gene dataset (18S + 28S + COI). Posterior probability support indicated at nodes. Black dots indicate full statistical support; values < 0.90 are not shown. Specimen vouchers and population names correspond to those reported in Table S1. Med = Mediterranean Sea; NS = North Sea; Hac = *Halichaetonotus aculifer*; Hat = *Halichaetonotus atlanticus*; Tbo = *Turbanella bocqueti*; Xin = *Xenotrichula intermedia*

Within *X. intermedia*, Mediterranean specimens (Xin_Med) are subdivided into two fully supported clades (pp = 1.00). One includes all specimens sampled from the Ligurian Sea (Med_Lig), while the other groups together specimens from the northern Adriatic Sea (Med_Adr).

DNA species delimitation

Xenotrichula intermedia

The 4× rule strongly supported the separation between Mediterranean (Med) and northern (NS) lineages, with K/θ values well above the threshold of 4 ($K/\theta \approx 44$), in agreement with the interpretation of these lineages as independently evolving units (Fig. 4A). This is consistent with the large divergence observed between these groups, with pairwise distances ranging from 0.2532 to 0.2805 and a substantial barcode gap of approximately 0.233 (calculated from a minimum inter-lineage divergence of 0.2532 and a maximum within-lineage variation of 0.0200), with no overlap between intra- and inter-lineage distances (see Supplementary Table S2).

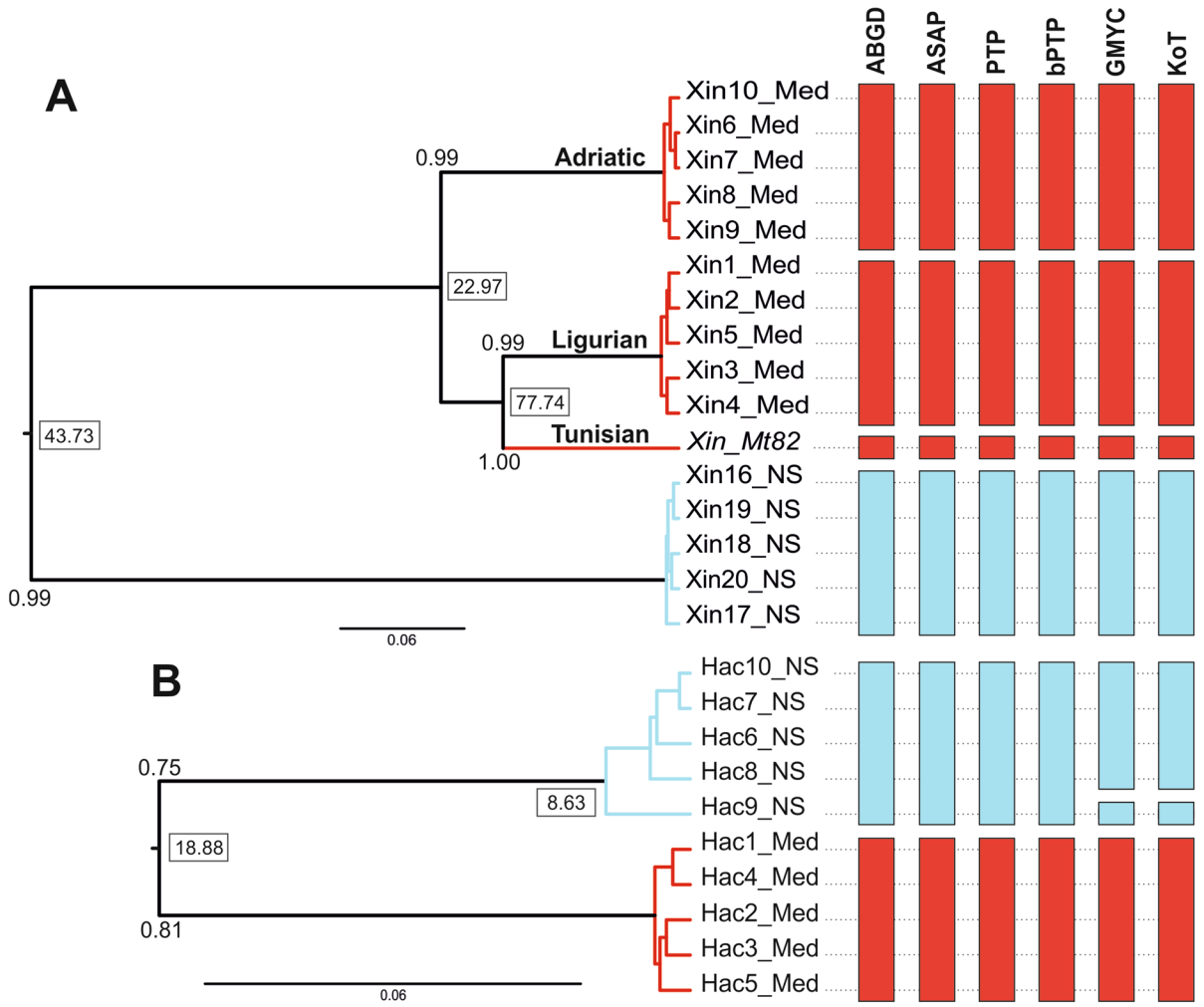


Fig. 4 Ultrametric trees generated with BEAST using the COI datasets. **A** *Xenotrichula intermedia.* **B** *Halichaetonotus aculifer*. Species-delimitation results are indicated by vertical bars below each tree; the Mediterranean and Northern lineages are color coded in both bars and branches. Posterior probabilities (≥0.7) from the PTP species delimitation analyses are shown at the nodes. K/θ values (≥4) between different branches are shown in boxed frames at the corresponding nodes. Sequences retrieved from GenBank are shown in italics

Within the Mediterranean, the 4× rule also supported the presence of two distinct lineages corresponding to the Ligurian (Med_Lig) and Adriatic (Med_Adr) populations. Distances between these groups ranged from 0.1494 to 0.1621, yielding a barcode gap of approximately 0.129 (0.1494 − 0.0200), again with complete separation from within-lineage variation and K/θ values well above the threshold.

The Tunisian sample (Xin_Mt82) occupied an intermediate position, showing divergence values of 0.1275–0.1403 from Med_Lig and 0.1530–0.1603 from Med_Adr. These correspond to barcode gap values of approximately 0.108 relative to Med_Lig (0.1275 − 0.0200) and 0.142 relative to Med_Adr (0.1530 − 0.0109), indicating that Xin_Mt82 represents an additional, distinct Mediterranean lineage.

Consistent with these results, ABGD analysis recovered stable partitions corresponding to the same lineage structure, separating the northern lineage from Mediterranean samples and further subdividing the latter into Ligurian and Adriatic groups, with Xin_Mt82 forming a distinct unit. Similarly, ASAP analysis identified the same partition as the

best-supported solution (score = 1), confirming the robustness of the inferred lineage structure.

Tree-based species-delimitation approaches consistently recovered four evolutionary lineages in the Xi dataset (Fig. 4A). Med_Lig, Med_Adr, NS samples, and Xin_Mt82 formed four well-defined and reciprocally distinct clusters, separated by long internal branches relative to the short branches observed within each group. Under a GMYC framework, this branching pattern is consistent with a clear transition from coalescent to speciation processes and supports a four-lineage model (maximum likelihood of GMYC model: 80.0401; maximum likelihood of null model: 70.5506; LRT: $P = 7.5635 \times 10^{-5}$; number of ML clusters: 3, confidence interval: 3–3; number of ML entities: 5, confidence interval: 5–5). Similarly, the PTP analyses, which rely on branch length differences, recovered the same four-lineage partition (support values 0.993–1.000).

The haplotype network analysis recovered a clear separation between the North Sea and Mediterranean populations (Fig. 6A; see Supplementary Table S3). The network further supported the internal structuring of the Mediterranean populations, distinguishing the Ligurian and Adriatic lineages, while placing the Tunisian specimen in close association with the Ligurian population (Fig. 6A).

Overall, all methods were fully concordant in recovering a clear separation between Mediterranean and North Sea lineages, as well as a hierarchical structuring within the Mediterranean region, including multiple independently evolving lineages.

Halichaetonotus aculifer

The 4× rule strongly supported the separation between Mediterranean and northern lineages, reaching a K/θ value well above the threshold of 4 typically used to delimit independently evolving lineages (≈ 19). Within the northern lineage, however, Hac9_NS showed greater genetic divergence from the remaining specimens ($K/\theta \approx 8$), indicating additional genetic structuring within that population.

Consistent with these results, ABGD analysis identified a primary partition separating Mediterranean and northern lineages, supported by high barcode gap values between these groups (0.119–0.151). Within the northern lineage, however, Hac9_NS showed markedly higher divergence relative to the remaining samples, resulting in a much smaller barcode gap (0.027) and indicating additional internal genetic structuring (see Supplementary Table S4).

ASAP analysis recovered the same primary partition as the best-supported solution, with the divergent position of Hac9_NS likewise indicating reduced cohesion within the northern lineage.

Tree-based species-delimitation methods consistently recovered the same two evolutionary lineages in the dataset (Fig. 4B). Hac_Med and Hac_NS samples formed two distinct clusters separated by a longer internal branch, whereas within-group branches were comparatively short. Within the northern lineage, specimen Hac9_NS was again recovered on a longer branch than the remaining northern specimens. Under a GMYC framework, this branching pattern supported the two-lineage model (maximum likelihood of GMYC model: 49.3511; maximum likelihood of null model: 45.8686; LRT: $P = 0.0307$; number of ML clusters: 2, confidence interval: 1–3; number of ML entities: 4, confidence interval: 2–7). The PTP analyses recovered the same two-lineage partition, with good support (0.751–0.818). Overall, the tree-based methods consistently recovered Hac_Med and Hac_NS as distinct evolutionary lineages.

The haplotype network recovered the distinction between the two populations as well, in full concordance with the results of the other approaches; additional genetic structuring is once again evident in the northern lineage, with one haplotype (corresponding to specimen Hac9_NS; see Supplementary Table S5) recovered as a distinct entity (Fig. 6B).

Halichaetonotus atlanticus

The 4× rule analysis resulted in a K/θ ratio of 4.48 between the northern and Mediterranean lineages, slightly above the threshold value of 4, providing only marginal support for the presence of two independently evolving lineages (Fig. 5A; see also Supplementary Table S6). Although this criterion is formally satisfied, the relatively small margin above the threshold suggests that this result should be interpreted with caution.

The ABGD analysis consistently produced a partition comprising six subsets, four of which contained

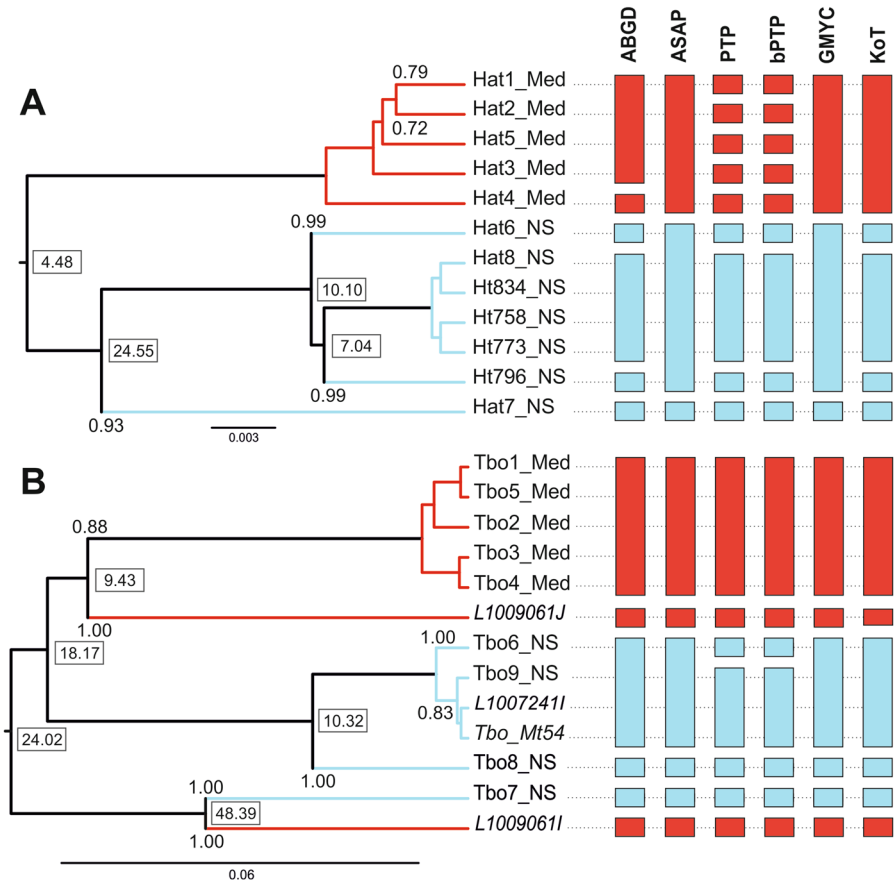


Fig. 5 Ultrametric trees generated with BEAST using the COI datasets. A. *Halichaetonotus atlanticus.* **B** *Turbanella bocqueti*. Species-delimitation results are indicated by vertical bars below each tree; the Mediterranean and Northern populations are color coded in both bars and branches. Posterior probabilities (≥0.7) from the PTP species delimitation analyses are shown at the nodes. K/θ values (≥4) between different branches are shown in boxed frames at the corresponding nodes. Sequences retrieved from GenBank are shown in italics

a single specimen (Hat4_Med, Hat6_NS, Hat7_NS and Ht796_NS), while the remaining subsets broadly corresponded to the Hat_Med and Hat_NS groups. However, the analysis did not identify a pronounced barcode gap. Similarly, ASAP recovered a best-supported partition (score = 1.5) comprising three lineages corresponding to the Mediterranean and North Sea groups, with specimen Hat7_NS recovered as a distinct entity (Fig. 5A).

Overall, the tree-based methods consistently recovered Hat_Med and Hat_NS as distinct evolutionary lineages, although with weaker statistical support than in the other investigated taxa. The PTP analyses grouped together the northern specimens Hat8_NS, Ht758_NS, Ht773_NS and Ht834_NS, while recovering the remaining specimens as separate entities; however, this reconstruction received overall low statistical support (<0.700). Likewise, the GMYC analysis recovered the same three lineages as ASAP, but the model itself did not reach statistical significance.

The haplotype network recovered a clear separation between North Sea and Mediterranean populations. The haplotype corresponding to specimen Hat7_NS is shown as a separate entity in the northern population (Fig. 6C; see Supplementary Table S7).

Turbanella bocqueti

The 4× rule supported the separation between a core Mediterranean lineage (Tbo1_Med–Tbo5_Med) and a core northern lineage including Tbo6_NS, Tbo9_NS, Tbo_Mt54 (Ireland), and L1007241I (Normandy), with K/θ ratios exceeding the threshold value of 4 (≈ 18) (Fig. 5B). This distinction is also reflected in a clear barcode gap between these groups (≈ 0.059). Consistent with these results, ABGD and ASAP analyses recovered a primary partition separating Mediterranean and northern lineages.

Within the northern lineage, Tbo7_NS and Tbo8_NS showed elevated divergence relative to the core NS lineage, indicating additional genetic structuring

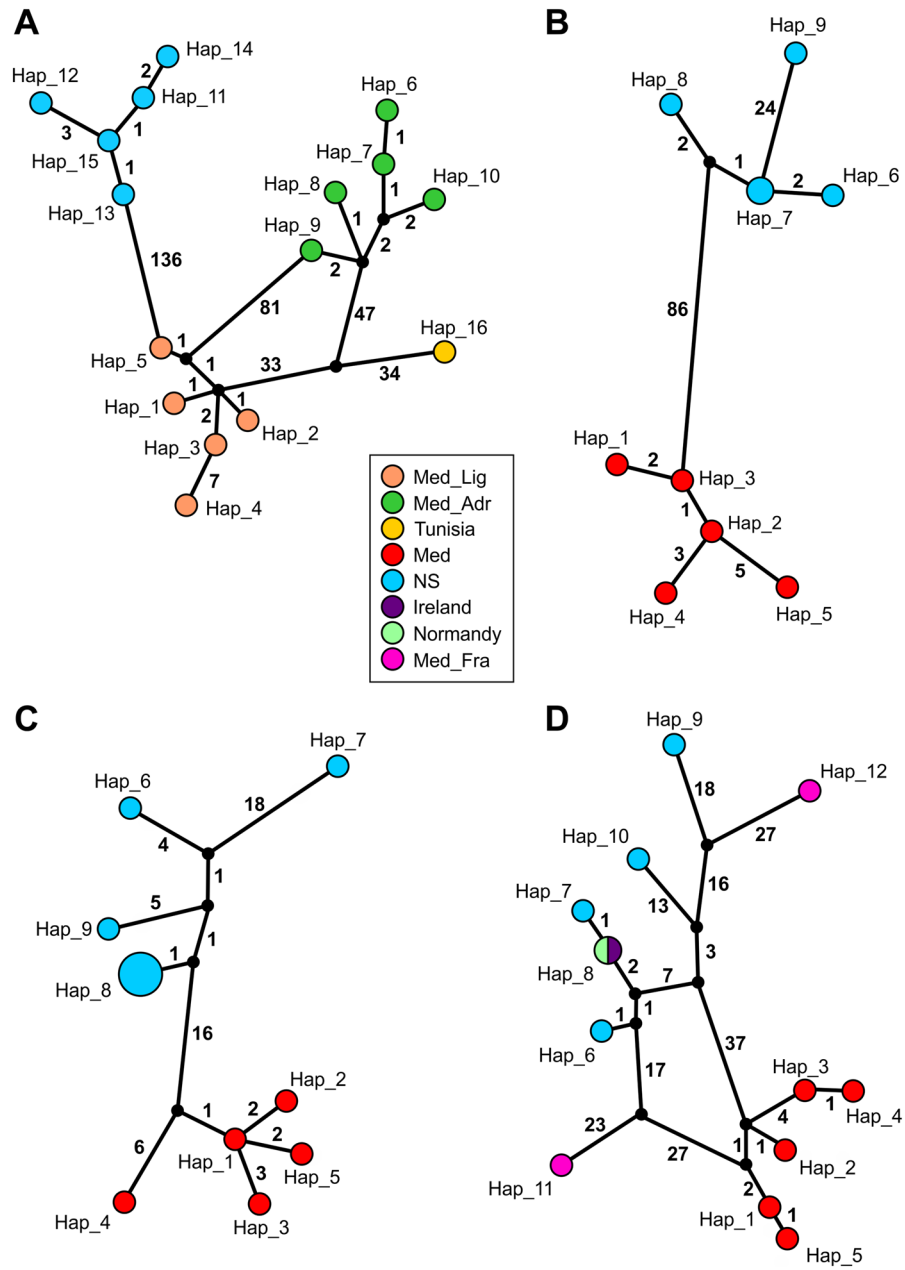


Fig. 6 TCS haplotype networks based on COI sequences of the investigated populations. **A** *Xenotrichula intermedia*. **B** *Halichaetonotus aculifer*. **C** *Halichaetonotus atlanticus*. **D** *Turbanella bocqueti*. Each circle represents a unique haplotype. Larger circles indicate haplotypes shared by multiple specimens. Colors indicate the geographic origin of the specimens. Numbers along the connecting lines indicate the number of mutational steps separating adjacent haplotypes. For graphical constraints, connecting-line lengths are not drawn to scale

among the northern specimens. Similarly, within the Mediterranean, the two specimens from southern France (Med_Fra; L1009061J and L1009061I) were distinct from both the core Mediterranean lineage and from each other (distance = 0.0884), suggesting additional lineage diversity within the Mediterranean basin (see Supplementary Table S8).

Distance-based delimitation methods (ABGD and ASAP) recovered the same six-lineage partition as the best-supported solution (ASAP score = 1). Tree-based approaches (GMYC/PTP-style interpretation) were fully concordant with these results (PTP support values 0.832–1.000; maximum likelihood of GMYC model: 57.9365; maximum likelihood of null model: 53.2419; LRT: $P = 0.0091$; number of ML clusters: 3, confidence interval: 2–3; number of ML entities: 4, confidence interval: 3–7) (Fig. 5B).

The haplotype network was consistent with both the phylogenetic reconstruction and the species-delimitation analyses, recovering two principal lineages. The North Sea lineage showed greater internal genetic diversity than the Mediterranean lineage. The two southern French specimens were recovered closer to the northern lineage than to the core Mediterranean lineage, while remaining clearly differentiated from both the northern specimens and from each other (Fig. 6D; see also Supplementary Table S9).

Overall, these analyses recovered multiple evolutionary lineages, with a primary separation between Mediterranean and northern European populations together with additional genetic structuring within both geographic regions.

Discussion

In this work, we examined a total of thirteen European populations belonging to four species and found previously undetected levels of hidden genetic diversity in the region, not reflected in the external morphology of these animals. Nine of the involved populations were sequenced for the first time, expanding the available molecular data for the corresponding taxa.

Across datasets, a consistent and prominent pattern is the genetic differentiation between Mediterranean (Med) and northern European (NS) samples, which are generally recovered as distinct evolutionary lineages. This pattern is most clearly illustrated by the *Xenotrichula intermedia* dataset. The multigene phylogenetic analysis recovered the Ligurian, Adriatic, and North Sea populations as three distinct evolutionary lineages. Notably, the Ligurian and Adriatic populations, despite their relatively close geographic proximity, formed two fully supported clades (Fig. 3).

This discovery contrasts with the previous hypothesis of a single pan-European *X. intermedia* taxonomic entity (Munter & Kieneke, 2017), indicating that *X. intermedia*, as currently recognized, is likely to represent a species complex. The congruence among p-distance analyses, the K/θ (4× rule), ABGD, ASAP, GMYC, and PTP provides strong molecular evidence that the Ligurian, Adriatic, and North Sea lineages are best interpreted as three distinct candidate species-level entities (Fig. 4A).

The inclusion of a Tunisian specimen in the COI-based species-delimitation analyses further expanded this scenario by identifying an additional Mediterranean lineage. The network analysis places the Tunisian specimen closest to the Ligurian lineage while maintaining its genetic distinctiveness, suggesting that it may represent an additional Mediterranean evolutionary entity, the significance of which requires further investigation.

The discovery of multiple genetically differentiated lineages also within the Mediterranean basin calls into question the cosmopolitan status traditionally attributed to *X. intermedia*. Future studies integrating broader geographic sampling with detailed morphological and molecular analyses will be necessary to clarify the distribution and taxonomic status of this species complex and to better assess its true biodiversity.

The Med–NS differentiation is also evident in the *Halichaetonotus aculifer* dataset, where Mediterranean and northern European samples are consistently recovered as two well-defined and reciprocally distinct evolutionary lineages across all analytical approaches. The divergence observed in this species is particularly intriguing, as *H. aculifer* possesses several morphological traits that have been considered conducive to long-distance dispersal. It is small (around 160–170 µm), parthenogenetic, and bears long, slightly flattened ventrolateral spines, which have been hypothesized to facilitate flotation and pelagic dispersal (Ruppert, 1977). Despite these features, all analyses consistently recover the North Sea and Mediterranean populations as two distinct evolutionary lineages.

Additionally, the results indicate additional genetic structuring within the North Sea population, with one investigated specimen (Hac9_NS) appearing differentiated from the remaining northern samples. Based on the currently available evidence, we interpret the observed differentiation of Hac9_NS as intraspecific genetic variation, given that the multilocus phylogeny places this specimen within the North Sea lineage and only two of the six species-delimitation approaches recover it as a distinct species. This interpretation is further corroborated by the network analysis, which places Hac9_NS within the North Sea group despite

its distinct position relative to the remaining northern specimens.

In line with this general pattern, the analyses of *Halichaetonotus atlanticus* and *Turbanella bocqueti* also revealed a differentiation between Mediterranean and northern European populations. However, they also highlighted a more complex genetic structure, with additional lineages recovered within one or both geographic regions.

The main set of Mediterranean *T. bocqueti* sequences (Tbo1–5_Med) forms a cohesive and consistently recovered core Mediterranean lineage, characterized by low internal genetic diversity and a strong separation from a geographically widespread northern lineage comprising samples from northern France and Ireland. This pattern indicates clear geographic structuring between Mediterranean and northern European populations. Remarkably, the pairwise distance analysis and the haplotype network show that the Irish specimen and the specimen from the coast of Normandy share the same COI sequence and, consequently, the same haplotype (see also Table S5). This observation could reflect ongoing genetic connectivity among northern European populations, extending from the North Sea to the Atlantic coast. However, this hypothesis requires confirmation through broader geographic sampling, as the observed pattern could equally reflect the limited resolution of the partial COI sequence, a recent range expansion of the species, or simply the consequence of sampling limitations.

Beyond this primary North Sea–Mediterranean contrast, additional genetic structuring was detected within *T. bocqueti*. Notably, the French Mediterranean specimens from the locality near Nice (Med_Fra) did not cluster within the core Mediterranean lineage (represented by specimens from the southern coast of Sicily), but instead formed a separate, well-supported lineage distinct from both the core Mediterranean and northern lineages. The two French *T. bocqueti* specimens, collected at the same locality and on the same date (Kieneke et al., 2012), are highly divergent and were never recovered together in any of the analyses. Similarly, a divergent lineage was identified among the northern samples (i.e., Tbo7_NS), further highlighting the complex genetic structure of this species.

These results are consistent with previous findings for the congeneric species *Turbanella hyalina*, which also exhibits high levels of internal genetic variability and includes at least two sympatric cryptic species in North Sea populations (Kieneke et al., 2012; Kieneke & Nikoukar, 2017). In *T. hyalina*, the authors suggested that this genetic differentiation may be driven by differences in the reproductive timing of the two cryptic species (Kieneke et al., 2012). One possible explanation for the differentiation observed in *T. bocqueti* is that it may be associated with the diversity of substrates colonized by these organisms, which, as previously noted, includes anoxic environments (Tongiorgi, 1975). However, the limited number of specimens and populations included in the present study, together with the lack of detailed ecological and life-history data, does not allow this hypothesis to be evaluated. These findings therefore provide a preliminary framework for future studies aimed at clarifying the phylogeography and taxonomy of the *T. bocqueti* complex.

In contrast to the patterns observed in the other taxa, the results for *H. atlanticus* remain ambiguous. Although they hint at a genetic partitioning between North Sea and Mediterranean lineages, this signal is comparatively weak and lacks robust statistical support in species-delimitation analyses, despite the clear separation between Mediterranean and North Sea populations recovered in the network analysis. The shallow divergence observed, coupled with the species' apparent rarity in the Mediterranean basin, may point to a relatively recent colonization event, with populations possibly still in the early stages of divergence. Additional sampling and integrative approaches will be essential to clarify the evolutionary history and geographic distribution of *H. atlanticus*.

Taken together, the concordant results obtained from the multilocus phylogeny and the six complementary species-delimitation approaches indicate that the recovered lineages are, in most cases, consistent with species-level differentiation rather than simply geographically structured populations. However, we refrain from formally recognizing and describing these entities as new species because this would require an integrative taxonomic revision based on molecular evidence, detailed morphological investigations, including methods capable of revealing characters not detectable using conventional

light microscopy, as well as ecological information where available. This is particularly important for lineages that did not receive full support in the multilocus phylogenetic analysis or that are currently represented by a single COI sequence. Such an integrative taxonomic revision was not envisaged in the experimental design of the present study and therefore falls beyond its scope. Nevertheless, the marked genetic differentiation detected between North Sea and Mediterranean populations is robust and corroborates patterns previously reported in the literature.

Our findings are consistent with the growing body of evidence demonstrating extensive cryptic diversity across marine meiofaunal taxa. Studies on a wide range of groups, including flatworms (Tessens et al., 2021), annelids (Cerca et al., 2020), nemerteans (Leasi & Norenburg, 2014), and rotifers (Fontaneto et al., 2010; Stec et al., 2026), have reached similar conclusions, showing that many nominally cosmopolitan species are more accurately interpreted as species complexes. Likewise, the occurrence of cryptic species endemic to the Mediterranean Sea is an established biogeographic feature among small-bodied taxa (Jörger et al., 2012; Tessens et al., 2021; Furfaro et al., 2022).

By contrast, the Holarctic region harbors taxa with broader geographic distributions, a pattern generally attributed to its comparatively stable ecological and climatic history (Kieneke et al., 2012; Worsaae et al., 2019; Radomski, 2025; Stec et al., 2026; Wernström et al., 2026). However, these widely distributed taxa frequently exhibit substantial intraspecific genetic structuring (Lindholm et al., 2016), indicating marked regional differentiation despite their extensive geographic ranges. These broader biogeographic patterns provide an independent context for interpreting the results of the present study.

Conclusions

Our study demonstrates a clear pattern of geographic structuring between northern European and Mediterranean populations in all four investigated morphospecies and reveals substantial, previously unrecognized lineage diversity in these taxa, particularly within the Mediterranean basin. Taken together, the concordant results obtained from the multilocus phylogeny and the complementary species-delimitation approaches indicate that, in three of the four investigated morphospecies, most recovered lineages are best interpreted as distinct candidate species-level lineages rather than simply as geographically structured populations. These findings are consistent with a contribution of historical geographic isolation and restricted gene flow to lineage divergence between northern European and Mediterranean populations, alongside additional regional diversification within the Mediterranean, although the historical and ecological processes underlying these patterns remain to be investigated.

These findings highlight the need for a systematic re-examination of the Mediterranean gastrotrich fauna. A substantial proportion of described marine gastrotrich species originates from northern Europe, and Mediterranean taxa have traditionally been identified by direct reference to those descriptions. The present results demonstrate that this approach is likely to overlook significant regional diversity. A systematic re-examination of the Mediterranean gastrotrich fauna using an integrative taxonomic approach is therefore warranted in light of its genetic distinctiveness, with re-descriptions undertaken where appropriate and supported by combined molecular, morphological, and ecological evidence.

At the same time, an important limitation must be acknowledged: no specimens from the type localities of the investigated species could be included in the present study, as molecular data for those populations are still lacking. Consequently, it is presently not possible to establish which of the recovered lineages correspond to the nominal taxa. Although this question remains unresolved, populations sampled from the same broad geographic regions as the type localities are expected to provide plausible representatives of the nominal taxa, pending direct molecular confirmation. Accordingly, lineages occurring outside these regions represent potential candidates for future formal description as new species. While the investigated specimens are consistent with current morphological diagnoses, the formal taxonomic recognition of the new species will require an integrative revision combining molecular evidence with detailed morphological investigations using approaches beyond conventional light microscopy (e.g. confocal or electron microscopy), together with ecological information where available.

The need for such an integrative taxonomic revision is not restricted to geographically distant populations, as demonstrated by the hidden diversity uncovered in the northern populations of *Turbanella bocqueti* and *Halichaetonotus atlanticus*, close to their respective type localities. Taken together, these findings demonstrate that hidden diversity in marine Gastrotricha is not restricted to geographically distant populations but also occurs in populations from the same broad geographic regions as the nominal taxa. This pattern reinforces the need for an integrative taxonomic reassessment of the European marine gastrotrich fauna and further challenges the expectations of the EiE paradigm.

To achieve a more comprehensive understanding of the evolutionary and biogeographic relationships among the European populations of the taxa investigated in the present study, future studies should first expand geographic sampling along the Atlantic coastline in order to bridge the current geographic sampling gap between the North Sea and the Mediterranean Sea. This effort should be complemented, whenever feasible, by targeted molecular sampling from the type localities or nearby populations, as well as by broader geographic coverage across other European regions. In the case of *Xenotrichula intermedia*, sequencing populations for which muscular architecture has already been described would be particularly valuable, as it would enable direct comparisons between genetic divergence and anatomical variation. More broadly, integrative approaches combining molecular, morphological, and ecological data will be essential to reveal the true extent of diversity concealed beneath the conservative external morphology of these minute organisms.

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Data availability The data supporting this study are available within the article; genetic datasets are available on FigShare database (https://doi.org/10.6084/m9.figshare.32252361) and have been deposited in the NCBI GenBank database.

Declarations

Conflict of interest The authors declare no conflict of interest.

Institutional review board statement Not applicable.

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