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A complex species complex: The controversial role of ecology and biogeography in the evolutionary history of *Syllis gracilis* Grube, 1840 (Annelida, Syllidae)

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Abstract

The cryptic diversity in the polychaete *Syllis gracilis* Grube, 1840, in the Mediterranean Sea was examined with an integrative morpho-molecular approach. Individuals of *S. gracilis* were collected at eleven Mediterranean localities to provide an insight into the role of brackish environments in inducing cryptic speciation. The examination of morphological features combined with a molecular genetic analysis based on a partial sequence of the *16S rRNA* gene highlighted discrepancies between morphological and molecular diversity. Morphological data allowed to identify a morphotype with short appendages occurring in coralline algae communities and another one with long appendages observed in brackish-water environments and *Sabellaria* reefs. Multivariate analyses showed that sampling localities were the greatest source of morphological divergence, suggesting that phenotypic plasticity may play a role in local adaptations of *S. gracilis* populations. Molecular data showed the occurrence of four divergent lineages not corresponding to morphological clusters. Different species delimitation tests gave conflicting results, retrieving, however, at least four separated entities. Some lineages occurred in sympatry and were equally distributed in marine and brackish-water environments, excluding a biogeographic or ecological explanation of the observed pattern and suggesting instead ancient separation between lineages and secondary contact. The co-occurrence of different lineages hindered the identification of the lineage corresponding to *S. gracilis* sensu stricto. The discrepancy between morphological and molecular diversity suggests that different environmental and biogeographic features may interact in a complex and unpredictable way in shaping diversity patterns. An integrative approach is needed to provide a satisfactory insight on evolutionary processes in marine invertebrates.

KEYWORDS

Mediterranean Sea, mitochondrial DNA, phenotypic plasticity, polychaetes, species complex

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1 | INTRODUCTION

With the progress of scientific knowledge and molecular techniques, the evidence of cryptic speciation in marine invertebrates has become overwhelming (Miglietta, Faucci, & Santini, 2011). Although the occurrence of cryptic species is known from the first half of the 20th century (Mayr, 1942; Sonneborn, 1939) and has been subject of important terminological and theoretical debates (Dobzhansky, 1972; Steyskal, 1972), this phenomenon revealed itself more widespread and frequent than previously thought, especially in marine invertebrates (Allcock et al., 2011; Nygren, 2014; Nygren et al., 2018), where species boundaries can be concealed by phenotypic plasticity, lack of morphological differentiation between reproductively isolated lineages, or high degree of morphological variation (Petraccioli, Guarino, Maio, & Odierna, 2010; Sanna, Dedola, Lai, Curini-Galletti, & Casu, 2011). Cryptic speciation was thought to occur mainly in species with low dispersal capability, such as those with direct development or short planktonic lifespan of larvae (Palumbi, 1994; Valentine & Jablonski, 1983). However, recent evidence showed that the occurrence of cryptic species is frequent in both low-dispersal (Iannotta, Toscano, & Patti, 2009; Sponer & Roy, 2002) and high-dispersal species (Goetze, 2003; Ladner & Palumbi, 2012). Therefore, life cycle and dispersal features are not sufficient to define the phenomenon of cryptic speciation. Barriers to gene flow are considered fundamental factors that can induce population differentiation, and, ultimately, cryptic speciation in marine invertebrates. In fact, the presence of extrinsic barriers to gene flow, represented by stretches of unsuitable habitat (Casu, Casu, Lai, Cossu, & Curini-Galletti, 2006), or by hydrodynamic fronts, unfavorable currents, and land barriers (Fernández, Heras, Maltagliati, Turco, & Roldán, 2011; Pannacciulli, Bishop, & Hawkins, 1997; Pérez-Losada, Guerra, Carvalho, Sanjuan, & Shaw, 2002), may restrict or prevent the passive dispersal of larval stages (Palumbi, 1994). In addition, sharp ecological breaks may lead to physiological, behavioral, and reproductive divergences that can strongly reduce gene flow among apparently adjacent populations (Cognetti & Maltagliati, 2000; Ferguson & Taggart, 1991; Maltagliati, Casu, & Castelli, 2004). From this perspective, brackish-water environments have been often considered evolutionary hotspots, where differentiation processes occur at a significantly higher rate compared with marine environments (Bilton, Paula, & Bishop, 2002; Cognetti & Maltagliati, 2000; Iannotta et al., 2009).

Among invertebrate taxa, polychaete worms are interesting models for the understanding of microevolutionary processes and cryptic speciation. The occurrence of cryptic species in polychaetes has been verified with molecular techniques for 86 nominal species, belonging to 30 families (Nygren, 2014). Among polychaetes, Syllidae represent the most numerous family, with approximately 700 described species within a wide range of morphological and ecological diversity (Musco & Giangrande, 2005), and some documented instances of cryptic speciation (Aguado et al., 2019; Álvarez-Campos, Giribet, San Martín, Rouse, & Riesgo, 2017; Álvarez-Campos, Riesgo, & Giribet, 2017; Maltagliati et al., 2000; Westheide & Schmidt, 2003). In the majority of cases, however, evolutionary processes underlying cryptic speciation are still poorly known, and complexity of differentiation patterns is expected. Maltagliati et al. (2000) firstly reported cryptic speciation in Syllidae through allozyme markers; these authors recognized two parapatric lineages within *Syllis gracilis* Grube, 1840, whose differentiation was attributed to the different habitat types (brackish-water vs. marine habitats). Furthermore, RAPD investigations on the interstitial Syllidae *Neopetitia amphophthalma* (Siewing, 1956) from the Atlantic, Mediterranean, and Red Sea highlighted remarkable within-species genetic variability that, however, was interpreted as variation occurring at subspecific level (von Soosten, Schmidt, & Westheide, 1998). Conversely, Westheide and Hass-Cordes (2001) and Westheide and Schmidt (2003) assigned the status of cryptic species to the several well-characterized allopatric lineages they found. Moreover, in a survey on cryptic diversity in the Canadian polychaetes Carr, Hardy, Brown, Macdonald, and Hebert (2011) identified in *Syllis alternata* Moore, 1908, and *Syllis elongata* Day, 1949, two complexes composed by two and five cryptic species, respectively. More recently, Álvarez-Campos, Riesgo, et al. (2017), Álvarez-Campos, Giribet, et al. (2017) showed the occurrence of several cryptic lineages within the allegedly cosmopolitan *S. gracilis* and *Trypanosyllis krohnii* Claparède, 1864. In particular, the study dealing with cryptic diversity within *S. gracilis* highlighted eight deeply divergent lineages belonging to two different clades. According to Álvarez-Campos, Riesgo, et al. (2017), different species, originally identified as *S. gracilis* in different areas, can be distinguished based on the degree of fusion between shaft and blade of compound chaetae, and on the pigmentation of live organisms. These authors identified three apparently allopatric Mediterranean lineages representing a clade, but the absence of topotypic material prevented the identification of *S. gracilis* sensu stricto. Moreover, different Mediterranean lineages were indistinguishable on the basis of the shape of modified chaetae and the live color (Álvarez-Campos, Riesgo, et al., 2017). Lastly, Aguado et al. (2019) highlighted a previously overlooked pseudocryptic diversity within the genus *Amblyosyllis* Grube & Ørsted in Grube, 1857, combining two mitochondrial and one nuclear gene with morphological data.

The main purposes of this study are to deepen the knowledge about cryptic diversity within Mediterranean lineages of *S. gracilis*, to test Maltagliati et al.'s (2000) hypothesis on habitat-mediated cryptic speciation, and to understand whether different lineages are distinguishable using different morphological characters. Moreover, phylogenetic relationships will be used to infer on processes and factors that impinged on the genetic architecture of this species complex.

2 | MATERIALS AND METHODS

A total of 142 individuals of *S. gracilis* were collected from eleven Mediterranean coastal localities, six of which were in the Ligurian-Tyrrhenian basin, two in the Sicilian Strait, and three in the Adriatic Sea (Table 1). Among the sampling localities, Orbetello Lagoon, Varano Lake, and Pialassa della Baiona are brackish-water habitats (sensu Cognetti, 1982), whereas the remaining localities are strictly

TABLE 1 Sampling localities, dates, and geographic coordinates of *Syllis gracilis* populations in the Mediterranean Sea

Code	Locality	Habitat	Date	Latitude (N)	Longitude (E)	GenBank codes
PAL	Palmaria Island (Ligurian Sea)	C	Jan-2013	44.041	9.837	MK533016–28
BOC	Boccale (Tyrrhenian Sea)	C	Nov-2012	43.475	10.330	MK533029–41
SSC	Sassoscritto (Tyrrhenian Sea)	C	Apr-2013	43.465	10.341	MK533042–58
CAP	Capraia Island (Tyrrhenian Sea)	C	Apr-2013	43.015	9.823	MK533071–76
ORB	Orbetello Lagoon (Tyrrhenian Sea)	B	Mar-2012	42.441	11.211	MK533059–70
PTO	Porto Torres (Tyrrhenian Sea)	C	May-2013	40.842	8.404	MK533077–92
EMI	Eraclea Minoa (Strait of Sicily)	S	Aug-2012	37.390	13.279	MK533093–99
DLU	Donnalucata (Strait of Sicily)	S	Aug-2012	37.653	14.641	NA
VAR	Varano Lake (Adriatic Sea)	B	Nov-2014	41.906	15.758	MK533100–07
PBA	Pialassa della Baiona (Adriatic Sea)	B	Jun-2013	43.497	12.265	MK533108–17
RAS	Raša River Mouth (Adriatic Sea)	C	Mar-2012	44.938	14.069	MK533118–28

Note: GenBank codes refer to accession number of newly sequenced taxa. Habitat legend: C = intertidal coralline algae; S = *Sabellaria* reef; B = brackish environment.
Abbreviation: NA, Not available.

marine environments. Among the marine localities, individuals from Eraclea Minoa and Donnalucata were collected on reefs built by the ecosystem engineer polychaete *Sabellaria alveolata*. Recently, these reefs were re-evaluated as habitat with great ecological value, hosting a variety of organisms, a part of which show a strict association with this habitat type (Bertocci et al., 2017). In agreement with the peculiarity of *Sabellaria* reefs, we considered these samples as different from the ones obtained from shallow assemblages dominated by coralline algae. All living material was obtained by scraping hard surfaces in the upper subtidal and sorted alive. *Syllis gracilis* individuals were fixed in 96% ethanol and preserved at −20°C.

2.1 | Morphological characterization

Since fixation in ethanol alters morphological characters, such as color patterns and shape of the soft parts, morphological characterization was accomplished by using the characters that are not affected by fixation and that are considered informative for taxonomy of Syllidae according to San Martín and Aguado (2012). The morphological characters used in this study were (a) length of the pharynx in number of segments occupied; (b) length of the proventricle in number of segments occupied; (c) length of the dorsal cirrus at chaetiger 10 in number of articles; (d) length of the dorsal cirrus at chaetiger 20 in number of articles; and (e) starting point in number of chaetigers of ypsiloid chetae, which are a diagnostic feature for this species. Quantitative measures were avoided because the contraction of the body after the fixation makes them unreliable. We also tested the usefulness of two ratios calculated on the basis of the previously reported characters, namely the ratio between pharynx and proventricle lengths, and the ratio between the cirrus at chaetiger 10 and the cirrus at chaetiger 20. The former measure is a useful diagnostic feature in several species of Syllidae (San Martín & Aguado, 2012), whereas the latter is important because in *S. gracilis*, and unlike several other *Syllis* spp., there is only a slight alternation between short and long cirri. Therefore, the number of articles in dorsal cirri of the anterior part decrease slightly proceeding toward the pygidium, but after the 20th chaetiger the number of articles becomes constant (San Martín, 2003).

2.2 | Morphological data treatment

To analyze data, we followed a double approach based on both univariate and multivariate statistics in order to assess differences among populations or among individuals from different habitat types. Pairwise *t* tests between population pairs were performed for each morphological parameter and for the two ratios calculated on their basis (McDonald, 2014) in order to evaluate differences at a single variable. To carry out these statistics, we used the function pairwise.t.test (R stats package) in the software R v. 3.1.1. (R Core Team, 2014). Possible combinations of morphological characters were investigated by combining them through multivariate analysis, using the program PRIMER-E v. 5 (Clarke & Warwick, 2001). Firstly, we calculated the matrix of the Manhattan distances between each specimen pair (we chose to include only the length of the dorsal cirrus at chaetiger 20, in order to avoid the doubling of the weight of one variable). Then, we used the Manhattan distance matrix to perform non-metric multi-dimensional scaling (nMDS) (Clarke, 1993). The significance of the identified differences among populations and among habitat types (brackish-water, coralline algae and *Sabellaria* reef) was assessed with the ANOSIM (Clarke, 1993).

2.3 | Molecular genetic characterization

Genomic DNA was isolated from two parapods of each *S. gracilis* individual using the Qiagen DNeasy tissue kit following the manufacturer's instructions; the remaining parts of the specimens were deposited in the polychaete collection of the Natural History Museum of the University of Pisa (MSNP; Table S1). A 472 bp long internal

portion of the mitochondrial gene coding for the *16S rRNA* (*16S*) was amplified using specific primers designed by the authors (Sys L—TATCCTGACCGTGCGAA, Sys H—TTGAGTCTAGTGCATCCCATA). Each 25 μl PCR mixture contained about 60 ng of genomic DNA, 0.2 μM of each primer, 1.25 U of SigmaTaqDNA Polymerase, 1 × reaction buffer, and 200 μM of dNTPs mix. The concentration of $MgCl_2$ was set at 3mM, and 12.5 μg of bovine serum albumin was added to the reaction mixture.

PCR amplification profile was set as follows: initial denaturing step at 94°C for 2 min; 35 cycles of denaturing at 94°C for 1 min, annealing at 48°C for 1 min, and extending at 72°C for 1 min 30 s, and a final extending step at 72°C for 5 min. For all PCR reactions, negative controls and replicates were included. Electrophoretic runs were carried out at 4 V/cm for 20 min on 2% agarose gels, made using 1 × SBA buffer with ethidium bromide (10 mg/ml) for staining of DNA fragments. PCR products were purified using ExoSAP-IT (USB Corporation) and sequenced using an external sequencing core service (Macrogen Europe). The mitochondrial region was sequenced in both forward and reverse directions, and the corresponding sequencing runs were repeated twice in order to verify the reliability of results. The PCR products did not show occurrence of aspecificity, excluding the possibility of multiple nuclear mtDNA-like sequences.

2.4 | Phylogenetic reconstruction

Overall, the *S. gracilis* DNA sequence dataset was composed of 121 individuals, 113 of which newly sequenced, and eight gathered from GenBank (accession numbers: KX280948–55). Amplicon length was 472 bp, and alignment was 473 bp due to an insertion at position 278 in two outgroup individuals (Table S2). Five Syllidae species have been included in the dataset as outgroup: *Ramisyllis multicaudata* (GenBank accession number: KR534502), *Typosyllis armillaris* (GB an: JF903727), *Syllis hyalina* (GB an: EF123818), *Syllis picta* (GB an: KX280933), *Syllis variegata* (GB an: EF123822), and *S. multicaudata* (GB an: KR534502). Sequences were edited in BIOEDIT v. 7.2.5 (Hall, 1999) and aligned with ClustalX v. 2.1 (Larkin et al., 2007). The program jModelTest v. 2.1.6 (Darriba, Taboada, Doallo, & Posada, 2012), based on the hierarchical likelihood ratio test, was used to assess the best model of evolution for the sequences under the Akaike and Bayesian information criteria. Both criteria were consistent in selecting the model TIM2 + G (Posada, 2003) as the best-fitting model of nucleotide substitution for the whole dataset. Phylogenetic relationships among taxa were investigated through Bayesian Inference (BI) by means of MrBayes 3.2.6 (Ronquist et al., 2012) setting as model parameters: NST = 4, rates = gamma, and ngammacat = 4. Two independent runs, each consisting of four Metropolis-coupled MCMC chains (one cold and three heated chains), were run simultaneously for 5×10^6 generations. Each chain was sampled every 1,000 generations to obtain 5,000 sampled trees. The first 1,250 sampled trees (25%) were discarded as burn-in, with the remaining 3,750 trees used to estimate the Bayesian posterior probability (PP) of tree nodes. The convergence of chains was checked through the average standard deviation of split frequencies, that should reach a value <0.01 at the end of the analysis (Ronquist et al., 2012), and through the potential scale reduction factor which should reach a value around 1 (Gelman & Rubin, 1992). A 95% statistical parsimony network analysis was performed using the software package TCS 1.21 (Clement, Posada, & Crandall, 2000), aimed at searching for possible disconnections between groups of individuals, further inferring the genetic relationships among the haplotypes. Gaps were treated as a fifth character state. Statistical parsimony is an alternative method for network construction that joins haplotypes within a parsimony connection limit, the latter being the maximum number of differences not due to reversion between haplotypes for which a 95% confidence exists.

2.5 | Species delimitation

The separation of the identified lineages at species level was tested using three different single-locus species delimitation methods. First, we applied the PTP (Poisson Tree Processes) model and its Bayesian implementation, the bPTP (Zhang, Kapli, Pavlidis, & Stamatakis, 2013). The PTP/bPTP model, based on the phylogenetic species concept, tests for a significant shift in the branching rate along a species tree, by using the number of substitutions to assess the speciation rate (Zhang et al., 2013). Runs were performed by means of the bPTP web server (available at http://species.h-its.org/ptp/) on the Bayesian phylogenetic tree, using default options and 5×10^5 MCMC generations. To test the reliability of results, each run was checked for convergence by visualizing the likelihood plot: If convergence occurred, the chain should stay at high-likelihood locations most of the time during the run. The other methods, ABGD (Automatic Barcode Gap Discovery) (Puillandre, Lambert, Brouillet, & Achaz, 2012) and NDT (Nucleotide Divergence Threshold) (Hebert, Cywinska, Ball, & deWaard, 2003), are based on genetic distances and do not consider phylogenetic relationships within the dataset. The ABGD method works on sequences in order to detect the barcode gap as the first significant gap beyond this limit and using it to partition the data (Puillandre et al., 2012). We used the ABGD online tool (available at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with the default settings. The correct species estimate was selected, as suggested by Puillandre et al. (2012), using the gene-specific priors for maximum divergence of intraspecific diversity, corresponding to $p = .01$. The NDT method works on sequences in order to ranks taxa into entities applying the fixed threshold of 2% given by Hebert et al. (2003) for DNA barcodes, using a pairwise Kimura (1980) two-parameter model (K2P) genetic distances matrix. Analysis was performed by means of a script (see Scarpa, Cossu, Delogu, et al., 2017 for details) written for the R statistical environment (available at https://cran.r-project.org/).

3 | RESULTS

3.1 | Morphological characterization

A total of 131 individuals were morphologically characterized from ten out of the eleven available localities; individuals from Raša

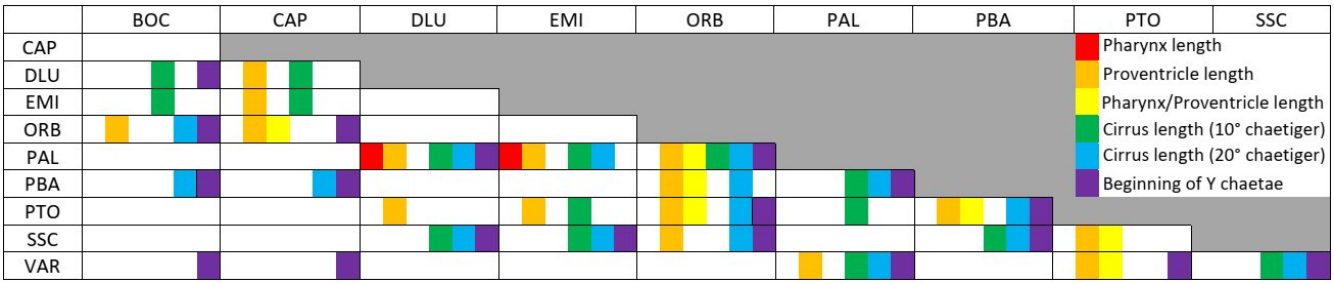

FIGURE 1 Diagrammatic representation of pairwise *t* test results for different morphological variables performed on different *Syllis gracilis* populations. Colored squares: significant; blank squares: non-significant. Population abbreviations are as in Table 1. The ratio between cirrus at chaetigers 10 and 20 did not show any significant variation between populations and therefore it was not reported

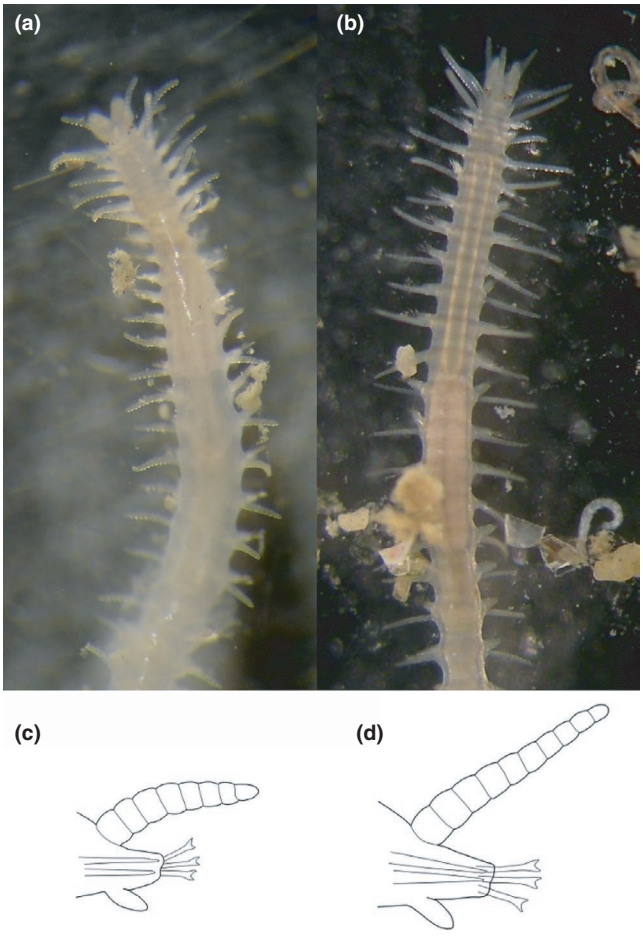

FIGURE 2 Comparison between the two morphotypes of *Syllis gracilis* identified from intertidal coralline algae (Sassoscritto: a, c) and in brackish environments (Orbetello Lagoon: b, d). (a, b) Anterior part of the animal in dorsal view; (c, d) 20th parapodium

River Mouth were not included in morphological analyses due to their preservation status (Figure 1). Pairwise *t* tests performed on each variable showed that a large part of the morphological variability occurs at population level (Figure 1). The lengths of pharynx and proventricle, the ratio between them, and the starting point of ypsiloid chaetae were significantly different between population pairs, but they did not show an association with geography or habitat type. The lengths of dorsal cirri at the 10th and 20th chaetiger showed instead a particular pattern, as all brackish-water population analyzed (Orbetello Lagoon, Pialassa della Baiona and Varano Lake) were significantly different from all marine ones, with the exceptions of Eraclea Minoa and Donnalucata. Based on these characters, it was possible to identify two divergent morphotypes. One morphotype was characterized by short dorsal cirri (7–10 articles at the 10th chaetiger, 7–8 articles at the 20th chaetiger), corresponding to specimens sampled at Boccale, Capraia, Palmaria, Porto Torres, and Sassoscritto, which are intertidal rocky marine environments dominated by coralline algae. The other morphotype had long dorsal cirri (12–14 articles at the 10th chaetiger, 10–11 articles at the 20th chaetiger) and corresponded to all brackish-water individuals and the marine form collected on *Sabellaria* reefs in southern Sicily (Figure 2). The ratio between dorsal cirri at the 10th and at the 20th chaetiger did not show any significant difference between populations; therefore, it was not reported in Figure 1. More generally, samples from Eraclea Minoa, Donnalucata, Orbetello Lagoon, Pialassa della Baiona, and Varano Lake represented a morphologically coherent group, as no significant differences among individuals were detected for any of the employed morphological variables. Similarly, the remaining samples showed a high degree of morphological homogeneity, with the exceptions of the significant difference in the beginning of ypsiloid chaetae between Palmaria and Porto Torres, and in the length of proventricle and in the ratio between pharynx and proventricle between Porto Torres and Sassoscritto (Figure 1).

Multivariate nMDS analysis produced a very good spatial ordination of individuals in a two-dimensional space, being the stress value (s = .16) abundantly lower to the threshold of reliability (s = .396 with 100 observations, Sturrock & Rocha, 2000). This analysis highlighted the occurrence of within-population morphological variability (Figure 3a), with a high degree of population overlapping. Moreover, populations showed a coarse clustering with regard to different environments (Figure 3b). In particular, individuals from brackish-water environments were different from those from coralline algae in the marine environment, even if a wide overlapping zone is observable. On the other hand, marine individuals collected on *Sabellaria* reefs and brackish-water individuals were intermingled and indistinguishable (Figure 3b). The ANOSIM carried out on the dataset confirmed this pattern, as the locality was identified as the main source of variation, (p = .001); also the habitat type represented a statistically

significant source of variation, with an average p = .021. However, pairwise ANOSIM tests showed that only the difference between marine individuals from coralline algae and those from *Sabellaria* reefs was statistically significant (p = .018), whereas the differences between both groups of marine populations and the brackish-water group were not. Also in this case, the analyses identified localities as the most important source of variation.

3.2 | Molecular genetic characterization

We obtained *16S* sequences for 113 individuals of *S. gracilis* from ten localities (Table 1) corresponding to 15 haplotypes (GenBank accession numbers: MK533016–MK533128). Unfortunately, it was not possible to amplify the *16S* region from the Donnalucata population as possible consequence of a high level of variation at the primers annealing region. Overall, the Bayesian phylogenetic tree identified four highly supported lineages (LG1, LG2, LG3, and LG4) within *S. gracilis*, also consistent with maximum parsimony network (Figures 4 and 5). Species tree showed the lack of geographic structure. In the haplotype network, the most represented lineage, LG1, included eight haplotypes and showed a star-phylogeny pattern, with a central haplotype shared by 63 individuals, and seven rare haplotypes separated by one or two mutations, except three haplotypes separated by the closest haplotype by seven, eight, and twelve mutations, respectively, and by one, four, and five mutational step from each other, respectively. The lineage LG2, including four haplotypes, was separated from LG1 by 30 mutations and showed a similar star-like pattern. LG3 was separated from LG1 by 28 mutations and included five haplotypes separated by one or two mutation from each other. The lineage LG4, grouping only sequences downloaded from GenBank, included three haplotypes that were separated from LG1 by 17 mutations (Figures 4 and 5). LG1 and LG2 included individuals from both marine and brackish-water environments, whereas LG3 included only marine specimens, with a strong prevalence of individuals collected on *Sabellaria* reefs. In LG1, brackish-water individuals represented a minor component, whereas in LG2 brackish-water and marine individuals were almost equally represented. Unfortunately, information about environmental features of the specimens obtained from GenBank was not sufficiently detailed to assign them to any of the considered habitat types, with the exception of the two sequences from *Sabellaria* reefs. At the population level, all localities except VAR included individuals belonging to two or even three lineages. However, populations often showed the prevalence of one lineage, whilst individuals belonging to other lineages were less frequent. Private haplotypes were found in single individuals of PAL, BOC, ORB, and EMI populations. Therefore, their absence from other populations might be due to sampling error.

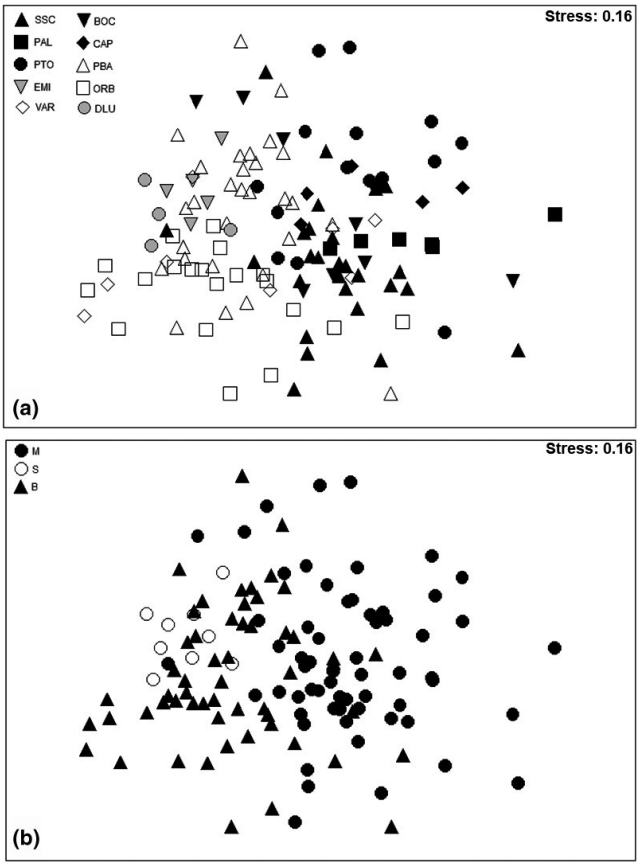

FIGURE 3 Non-metric multi-dimensional scaling pairwise morphological similarities between *Syllis gracilis* individuals. Individuals are distinguished based on the sampling locality (a) or habitat type (b). Population abbreviations are as in Table 1; B, brackish-water; M, marine on coralline algae; S, marine on *Sabellaria* reef

Species delimitation methods produced some differences in the total number of entities detected. The PTP/bPTP model identified a total of 10 entities, five of which were represented by clusters and five by a singleton (the multi-outgroup). The ABGD method, checked at the prior maximal distance (p = .01), identified 11 entities (five of which represented by the multi-outgroup). The NDT method found nine (five of which represented by the multi-outgroup), consisting of the lineages depicted by both species tree and haplotype network (Figures 4 and 5).

4 | DISCUSSION

Environmental features seemed to impinge on morphological characters of the *S. gracilis* complex. The univariate analysis of morphological characters showed that marine specimens sampled on intertidal algal communities are clearly differentiated from brackish-water and *Sabellaria*-related individuals. In particular, individuals occurring on intertidal algae are characterized by cirri that are shorter than those of brackish-water and *Sabellaria*-related individuals. Both univariate and multivariate analyses, however, identified the locality as the major factor of morphological variation in *S. gracilis*. The distribution of morphological variability, however, did not match the molecular diversity pattern. DNA sequence data allowed to identify four divergent mitochondrial lineages within the Mediterranean *S. gracilis*. Invertebrates often show discrepancies among different methods of species delimitation (Fontaneto, Flot, & Tang, 2015; Mills et al.,

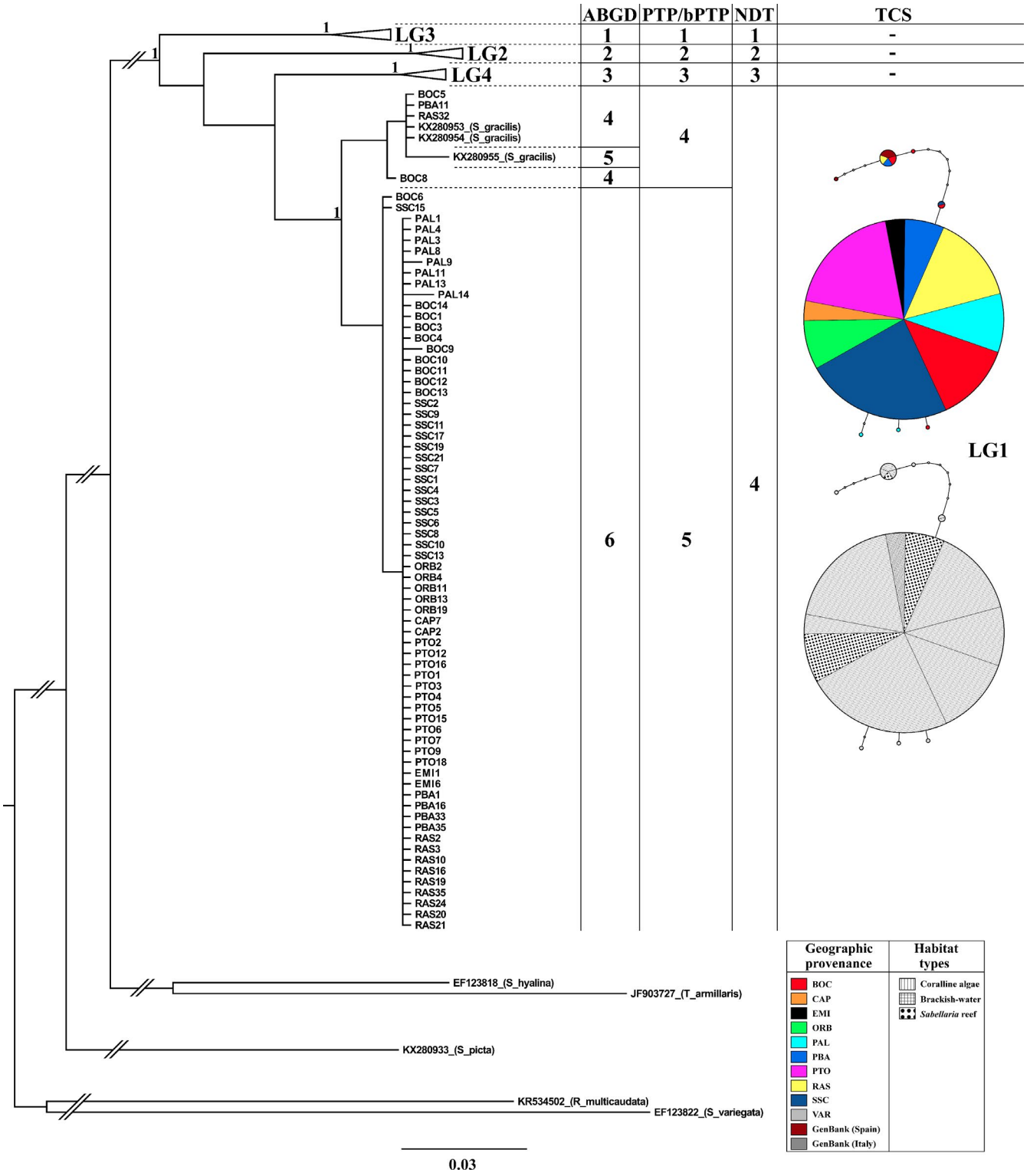

FIGURE 4 Bayesian phylogenetic tree, results of species delimitation methods and haplotype networks based on *16S* sequences. In the phylogenetic tree, clades LG2, LG3, and LG4 have been collapsed and are fully showed in Figure 5. The scale of branch length refers to the number of substitutions per site, and nodal supports are indicated as posterior probability for the main clades. Specimens with the same number within the same column have been ascribed to the same candidate species. Note that numbers across columns and thus across methods do not represent the same candidate species. Network colors indicate geographic provenance (above) and habitat types (below) according to the legend at the bottom right. Circle areas are proportional to haplotype frequency. Small white circles represent intermediate (unsampled or not existent) haplotypes. Each branch represents one mutational step

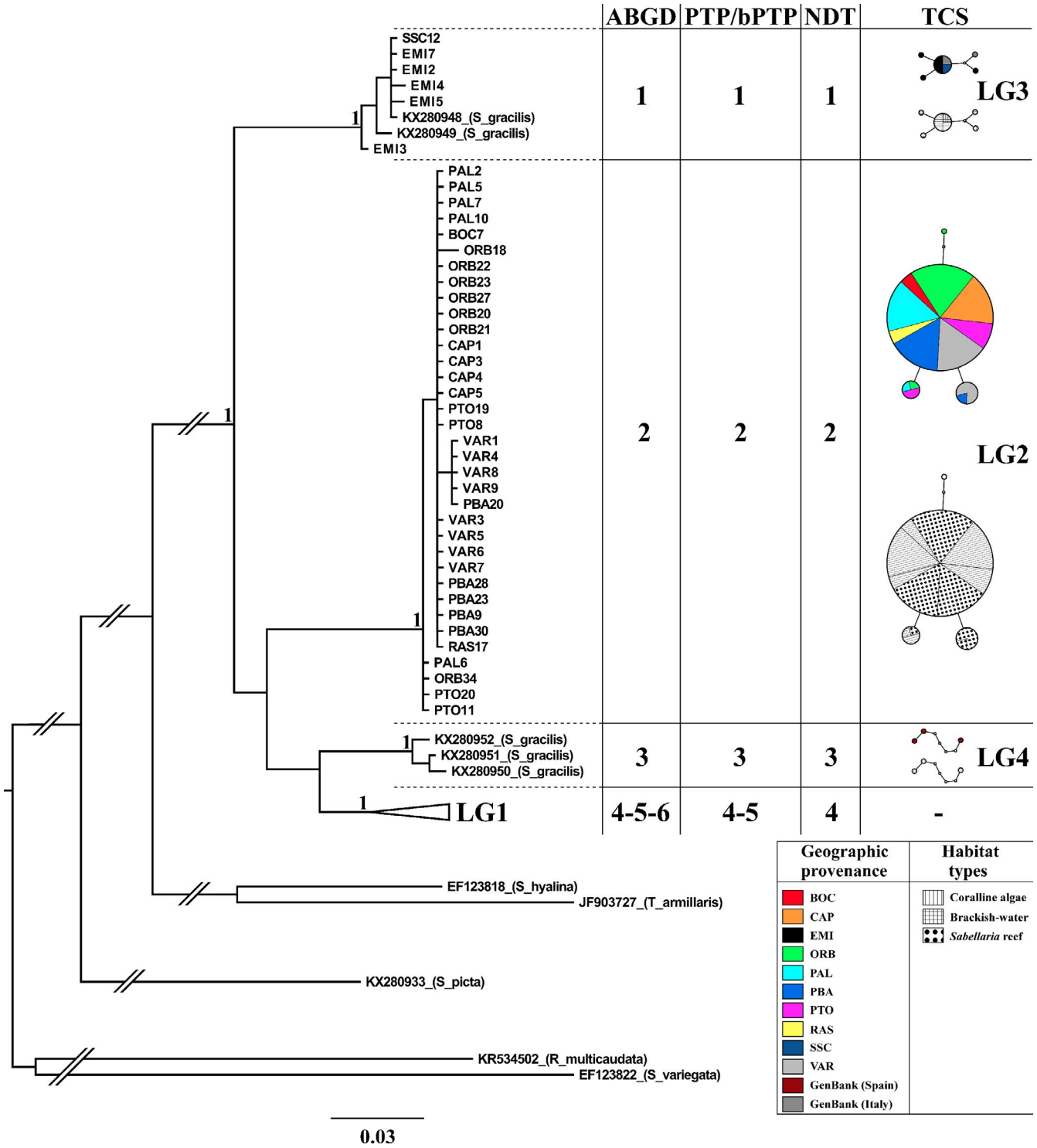

FIGURE 5 Bayesian phylogenetic tree, results of species delimitation methods and haplotype networks based on *16S* sequences. In the phylogenetic tree, clade LG1 has been collapsed and is fully showed in Figure 4. The scale of branch length refers to the number of substitutions per site and nodal supports are indicated as posterior probability for the main clades. Specimens with the same number within the same column have been ascribed to the same candidate species. Note that numbers across columns and thus across methods do not represent the same candidate species. Network colors indicate geographic provenance (above) and habitat types (below) according to the legend at the bottom right. Circle areas are proportional to haplotype frequency. Small white circles represent intermediate (unsampled or not existent) haplotypes. Each branch represents one mutational step

2017; Scarpa, Cossu, Delogu, et al., 2017; Scarpa, Cossu, Sanna, et al., 2017; Tang et al., 2012), especially comparing methods based on different criteria (i.e., PSC-based vs. genetic distance-based methods) (Scarpa et al., 2016). In this case, only NDT identified four entities corresponding to the four lineages identified by both the Bayesian phylogenetic reconstruction and maximum parsimony

haplotype network, whereas PTP/bPTP identified five entities, and ABGD six. Results from Bayesian phylogenetic reconstruction, TCS, and NDT are consistent with results of Álvarez-Campos, Riesgo, et al. (2017) who identified three divergent Mediterranean lineages within *S. gracilis* complex (here LG1, LG3, and LG4). The additional lineage identified (LG2) does not include published sequences and represents the result of the wider sampling plan. Consistently with Álvarez-Campos, Riesgo, et al. (2017), we retrieved discrepancy between the results of different species delimitation tests, that made uncertain an univocal identification of different cryptic species. This condition is quite common when methods based on different criteria and modeling assumptions are compared. Parsimony network implemented in TCS (Clement et al., 2000) and NDT method (Hebert et al., 2003) work following comparable ways, because they are not based (or affected) by evolutionary models. Nevertheless, the convergence of results obtained by BI species tree, parsimony network, and NDT is very interesting, because it highlights the quality of the DNA sequence dataset. The extent of the barcode gap employed by ABGD to detect entities depends on the variability of the dataset (Puillandre et al., 2012). In this case, several point mutations account for the high number of entities evidenced by ABGD. The PTP/bPTP analyses identify species boundaries based on the number of substitutions in the tree and may be affected by the unbalanced number of species (Zhang et al., 2013). In addition, the shallow phylogeny we obtained, showing a few deep branches, may cause oversplitting of the tree and consequent overestimation of the number of entities. Therefore, the interpretation of results requires a critical evaluation independently of the adopted species delimitation method, highlighting the absence of a passepartout key criterion for species identification. In order to maintain a conservative approach, we adopted the interpretation proposed by more than one method (BI species tree, TCS, and NDT) which converged in indicating the occurrence of four entities. Accordingly, distances retrieved between different lineages are slightly higher than those retrieved in *16S* sequences between different species of *Amblyosyllis* (Aguado et al., 2019), thus supporting the hypothesis of four cryptic species.

The absence of a correspondence between morphological and genetic clusters suggests the occurrence of phenotypic plasticity in the *S. gracilis* complex. Phenotypic plasticity implies the occurrence of different phenotypes as a response to different environmental pressures, in the absence of genetic divergence (Forsman, 2015; Whitman & Agrawal, 2009). This phenomenon is widespread across invertebrate phyla (Fusco & Minelli, 2010), but in polychaetes it is still scarcely studied, and in particular, while the identification of divergent phenotypes is rather easy, it is difficult to identify specific causes leading to divergent morphological adaptations (Meyer, Bleidorn, Rouse, & Hausen, 2008; Syomin, Sikorski, Bastrop, & Köhler, 2017). We suggest that phenotypic plasticity can be a suitable explanation for the morphological differences detected at among-habitat level. In particular, the shorter cirri detected in populations sampled on intertidal coralline algae might represent an adaptation to the higher wave exposure occurring in this environment, where shorter appendages may limit damages due to mechanic stress. Differences detected in other morphological features are more difficult to explain, but might be due to local adaptations.

While the majority of cases of cryptic speciation are identified between groups of organisms occurring in different geographic areas (von der Heyden et al., 2011; Solé-Cava, Klautau, Boury-Esnault, Borojecic, & Thorpe, 1991), or showing different ecological requirements (Bickford et al., 2007; Hyde, Kimbrell, Budrick, Lynn, & Vetter, 2008; Trabelsi et al., 2002), a striking peculiarity of *S. gracilis* is the sympatric occurrence of divergent mitochondrial lineages. In fact, individuals sampled in homogeneous environments of all localities belonged to two or three lineages, with the exception of the population of Varano Lake that included only individuals belonging to one lineage (LG2). The identification of a relationship between clades and geographic areas appears therefore impossible, as all clades are spatially widespread, and occur in both the Western Mediterranean (Tyrrhenian Sea) and Adriatic Sea. Only one lineage (LG3) is in strict relationship with *Sabellaria* reefs in the Strait of Sicily, even if one individual belonging to this lineage has been also recorded in the northern Tyrrhenian Sea. As *Sabellaria* reefs are known to host several species that are strictly related to this habitat type (Bertocci et al., 2017), the hypothesis of a *Sabellaria*-related lineage of the *S. gracilis* complex cannot be excluded. The sole biogeographic boundaries in the Mediterranean Sea, thence, cannot explain the divergence between different lineages of the *S. gracilis* complex. On the other hand, the hypothesis of divergence mediated by habitat type advanced by Maltagliati et al., (2000), is not corroborated by our results. In fact, LG1 and LG2 included brackish-water individuals with similar frequencies, and their absence in LG3 did not allow to formulate an unambiguous explanation, as this pattern could be the consequence of ecological or biogeographic processes, or even due to genetic drift. This is rather surprising, as brackish environments are commonly identified as habitats promoting the diversification of lineages and enhancing the rate of speciation processes (Cognetti & Maltagliati, 2000; Pereyra, Bergström, Kautsky, & Johannesson, 2009; Trabelsi et al., 2002). Speciation in brackish environment is enhanced by the strong environmental pressures, that on one hand cause the rapid arise of specific adaptations, and quick genetic drift in brackish populations (Iannotta et al., 2009; Trabelsi et al., 2002), and on the other hand reduce fitness of marine individuals, strongly reducing their contribution to the overall genetic diversity, and thus greatly restricting gene flow (Svensson et al., 2017). However, the occurrence of reproductive dispersal phases in *S. gracilis* complex (San Martín, 2003) might effectively counteract the genetic differentiation between brackish-water and marine individuals observed in many marine invertebrates lacking of dispersal stages in their life cycle (Cognetti & Maltagliati, 2000). Moreover, the occurrence of at least two lineages in brackish-water localities suggests an adaptability to brackish environments of the whole complex.

In the case of the *S. gracilis* complex in the Mediterranean Sea, therefore, molecular diversity did not show a clear relationship with geographic or ecological barriers—with the possible exception of LG3, that may be related to *Sabellaria* reefs—while morphological

diversity is clearly unrelated with geographic factors and seems to depend mostly on environmental features. A possible explanation for the divergence observed between sympatric lineages of *S. gracilis* could take into account past geological events separating groups of organisms that only secondarily came back in contact. This phenomenon is already known for Mediterranean populations of invertebrates and vertebrates (Albaina, Olsen, Couceiro, Ruiz, & Barreiro, 2012; Angiulli, Sola, Ardizzone, Fassatoui, & Rossi, 2016). In species with relatively low dispersal capabilities such a scenario would imply a strongly uneven geographic distribution of different genotypes (Albaina et al., 2012), whereas a completely overlapping distribution of divergent genotypes has been observed especially in demersal fishes, where few generations are enough to spread a new genetic variant throughout the whole distributional range of the species (Angiulli et al., 2016). Although dispersal estimates are not available for Syllidae, chiefly due to the scarcity of molecular studies on this group, the presence of a pelagic reproductive stage, as well as the ineffectiveness of the transition between brackish-water and marine environments as barrier to gene flow that can be inferred from our results, suggests that the pattern observed in *S. gracilis* might be closer to that of demersal fishes and that the apparent contradiction between the occurrence of deeply divergent clades and their almost perfect overlapping throughout the whole Mediterranean Sea might be at least partially explained by the hypothesis of a secondary contact between originally separated genetic clusters.

In several cases, the study of species complexes is aimed at (a) reconstructing which actual organisms correspond with the historical original descriptions, on the basis of type or topotypic material; and (b) resurrecting and re-evaluating previously synonymized taxa (Álvarez-Campos, Giribet, et al., 2017; Nygren & Pleijel, 2011). In this case, the identification of a lineage corresponding to Grube's (1840) original description of *S. gracilis* is currently impossible. The common co-occurrence of different lineages in the same locality suggests instead that Grube's material itself might have included more than one lineage. The list of synonymies stated by Fauvel (1911; 1923) for *S. gracilis* is clearly excessive, and it is not unlikely that names referred to Indo-Pacific material will be resurrected, especially in light of fine but stable morphological differences identified between Indo-Pacific and Atlantic-Mediterranean material traditionally referred to *S. gracilis* (Álvarez-Campos, Riesgo, et al., 2017). Conversely, Álvarez-Campos, Riesgo, et al. (2017) stated that different Mediterranean lineages are impossible to distinguish based on morphological features; our data corroborate their finding, highlighting in addition the presence of phenotypic plasticity that further conceals informative morphological differences. Previous studies suggested that some morphological characters, traditionally considered informative in polychaete taxonomy, might actually be affected by a remarkable degree of intraspecific variation (Meyer et al., 2008; Syomin et al., 2017). Our work suggests that genetic drift and local adaptations can further contribute to the structuring of patterns of morphological diversity. Morphological data should be therefore considered with caution in species diagnosis, taking into account the possibility of a wide intraspecific variability, and of population-specific adaptations.

In the case of *S. gracilis*, then, we are compelled to admit the extreme difficulty of solving the species complex. The discrepancy between morphological and molecular diversity patterns and the puzzling distribution of genetic variation may appear in some way confusing. Nonetheless, the *S. gracilis* case study represents a good example of the irreducible complexity within living systems, showing that different drivers affect different characters in different ways, ultimately leading to complex diversity patterns. The interaction between environmental and biogeographic factors is often unpredictable. In this work, the sole morphological results could have identified two groups, separated by environmental features, while through DNA sequence data we could have identified four groups or more. This inconsistency shows that the evolutionary history of the *S. gracilis* species complex is not driven by a single environmental or biogeographic feature; instead, it represents the result of long, unpredictable interactions among different evolutionary forces. This case study represents a *caveat* toward studies employing only molecular or morphological data to unravel biodiversity patterns, confirming that a satisfactory understanding of evolutionary processes can only be drawn from an integrative approach linking different types of data (Schlick-Steiner et al., 2010).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 List of *Syllis gracilis* specimens examined in this study, with respective voucher number in the MSNP collection, codes used in the paper and GenBank accession numbers.

Table S2 Complete alignment of 16S sequences employed in genetic analyses.

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