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Worming its way into Patagonia: an integrative approach reveals the cryptic invasion by *Eulalia clavigera* (Annelida: Phyllodocidae)

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Abstract

A phyllodocid polychaete belonging to the genus *Eulalia* is reported from Nuevo Gulf, Patagonia (South-western Atlantic Ocean) with abundant populations thriving in the intertidal zone. Morphological and molecular data allowed assigning this population to *Eulalia clavigera* (Audouin & Milne-Edwards, 1834), a species typically occurring along the north-eastern Atlantic coast. The absence of genetic structuring between north-eastern and south-western Atlantic *E. clavigera* strongly supports a non-native origin of the Patagonian population. Conversely, the majority of the Mediterranean *Eulalia* cf. *clavigera* analysed in this study turned out to belong to a different, probably undescribed species, suggesting that the diversity and taxonomy of green *Eulalia* is more complex than previously supposed. The high adaptation capabilities to stressed environments showed by *E. clavigera*, along with its possible high impact on native assemblages through predation, compel to carefully monitor its spread along the Patagonian coasts.

Keywords Non-native species · Phyllodocidae · Predation · Cryptogenic species · Species complex · Molecular characterisation

Introduction

The introduction of non-native species represents a major threat to natural ecosystems, especially when the introduced forms become invasive and eventually affect ecosystem functioning and human activities (Vilà et al. 2010), thus producing relevant economic losses (Pimentel et al. 2001). In the last decades, biological invasions in marine environments have dramatically increased, chiefly due to the technical and logistic improvements of maritime trade and the development of the transport network (Hulme 2009). The effects of non-native species colonising a new environment are often unpredictable, and even in the instance of an initial economical gain, the invasion process has usually a negative ecosystem impact, possibly leading to a complete change in natural assemblages (Molnar et al. 2008; Jeschke et al. 2014). For this reason, monitoring of biological invasions is essential for environmental management, allowing to plan impact reduction and, where possible, mitigation strategies (Wittenberg and Cock 2001). Monitoring plans aimed at early tracking of non-native species should concentrate on some areas that are

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particularly prone to biological invasions, among them, aquaculture facilities and commercial ports, as well as artificial canals connecting different basins or oceans (Molnar et al. 2008). However, the study of biological invasions in marine ecosystems is greatly hindered by the lack of knowledge regarding a large part of geographic areas. In particular, a close monitoring of species, of their spreading and of their impact on native communities is commonly realised only in European waters (Leppäkoski et al. 2002), in the Mediterranean Basin (Occhipinti-Ambrogi and Savini 2003), along the North American shores (Carlton 1989; Lodge et al. 2006) and in Australia (Pollard and Hutchings 1990a, b), whereas in other geographic areas the knowledge about non-native species is fragmentary and most likely their impact is greatly underestimated (Rilov and Crooks 2009). Moreover, even in well-monitored geographic areas, the majority of studies focus on a few taxa, such as fishes, crustaceans and molluscs, whereas information about the occurrence of the majority of non-native forms belonging to other phyla is anecdotic and fragmentary, and their effects on ecosystem functioning are scarcely known (Marchini et al. 2015).

In vertebrates and a few invertebrate groups, such as insects, echinoderms, or molluscs, it is relatively easy to assess whether a species is native or not. Instead, for several taxa, this question is far more complex to tackle: species with uncertain or unknown origin are considered cryptogenic and are particularly common in unicellular eukaryotes as well as in several macroinvertebrate groups, chiefly annelids and amphipods (Carlton 1996). This poses an additional difficulty to alien species management, in particular in marine environments (Carlton 1996). Even if cryptogenic species are usually treated as non-native in environmental management (Ojaveer et al. 2013), issues related to the interpretation of newly reported species may have consequences on environmental management practices, and on ecological status assessment in marine environments (Borja et al. 2005; Vilà et al. 2010). Thus, works aimed at solving cryptogenic species issues have high relevance.

Among marine animals, polychaete worms are characterised by a high frequency of cryptogenic species (Carlton 1996). This is due to a poor understanding of the diversity of some groups, which may lead to misidentifications, the inclusion of different biological species under the same name, and lack of information about the biogeography of several species (Giangrande 2003; Çinar 2013). Fifty out of the 292 alien polychaete species listed by Çinar (2013) are considered cryptogenic, at least in some areas; this high percentage (approximately 17%) shows that the occurrence of cryptogenic species in polychaetes is substantial. Moreover, alien and cryptogenic species are not evenly distributed among polychaete families. According to Çinar's (2013) review, groups that have been subject of thorough taxonomic revisions, such as Nereididae, Sabellidae and Serpulidae, show a high occurrence of alien species, and conversely, a lower number of cryptogenic ones, whereas groups with a more uncertain taxonomy, and putative occurrence of cryptic species, such as Spionidae, comprise a relatively low number of confirmed alien species, and a higher number of cryptogenic ones. Molecular techniques are often necessary to unravel the distribution and dispersal path of alien polychaetes (Blank et al. 2008), as well as the occurrence of cryptic species within traditionally recognised morphospecies with a wide geographic range (Barroso et al. 2010; Carr et al. 2011; Nygren 2014). Cryptogenic species issues can be solved with the appropriate use of such techniques as well (Blakeslee et al. 2008; Bolton et al. 2011), even though they have rarely been used to clarify the status of cryptogenic polychaete species (Carrera-Parra and Salazar-Vallejo 2011; Sun et al. 2017). A number of non-native polychaetes are known to have a strong impact on native communities. This is particularly evident for non-native polychaetes that are also ecosystem engineers and are known to strongly alter the invaded habitats and induce dramatic structural and functional changes in the native assemblages (Crooks 2002; Holloway and Keough 2002; Orensanz et al. 2002; Tovar-Hernández et al. 2011), but polychaete species might also have strong impacts on native assemblages by competing with, and eventually replacing, native species (Çinar and Altun 2007). Phyllodocidae are relatively large polychaetes that commonly occur in shallow-water assemblages; as the majority of them are carnivores or scavengers, they are expected to have a strong impact on the overall assemblages of colonised environments. In fact, Schimmenti et al. (2016) and Bertocci et al. (2017) suggest that the phyllodocid *Eulalia ornata* Saint-Joseph, 1888, occurring at high densities on Mediterranean *Sabellaria* reefs, may have a remarkable effect on ecological dynamics of this habitat through predation on other invertebrates.

In recent years, a phyllodocid polychaete belonging to the genus *Eulalia* has been reported with remarkably high densities on shallow rocky environments along the Patagonian coast in South America. In Patagonia, this homogeneously green phyllodocid was found crawling on rocks or in tide pools and Lobo Orensanz, who participated in the collecting trip, indicated he had not seen the species during the 1970s when he collected polychaetes along Argentinian shores (J.M. Orensanz pers. com. to MED and SSV, 2013). The species had been preliminarily identified as an undescribed species belonging to the *Eulalia viridis/clavigera* complex, but its sudden appearance along the Patagonian coast raised doubts about the native status of the species. This work has the purpose to clarify the identity of the *Eulalia* species spreading in Patagonia, to assess its eventual non-native status and to understand and discuss possible introduction paths and vectors.

Material and methods

Specimens of *Eulalia* sp. from Patagonia were collected at tide level in environments along the coast of Puerto Madryn (42°49.79′S; 64°53.1′W) in localities Cerro Avanzado, Punta Cuevas and Ambrosetti as indicated below (Fig. 1), during several years (2007–2013). Material for comparison from European waters was collected at tide level among mussels in Plymouth (50°40.45′N; 4°27.12′W) NW Atlantic during years 2006 and 2011 and at 0.2–0.5 m depth among calcareous algae at Capraia Island (43°1.77′N; 9°50.61′E), Mediterranean Sea, and in the port of Stintino, N Sardinia (40°56.13′N; 8°13′47.86″E), Mediterranean Sea, during year 2013 (Fig. 1). Along with this material, we studied also other museum specimens identified as *Eulalia clavigera* (Audouin & Milne-Edwards, 1834), collected in Bretagne, France, in 1994, and in Brazil in 2008. The examined material has been preserved in the institutional collections of El Colegio de la Frontera Sur, Chetumal, México (ECOSUR) and of the Laboratorio de Parasitología of the Instituto de Biología de Organismos Marinos, Puerto Madryn, Argentina (CNP), in the Los Angeles County Natural History Museum (LACM) and in the Natural History Museum of the University of Pisa, Italy (MSNP).

Specimens from Cerro Avanzado (Puerto Madryn), Plymouth, Capraia Island and Stintino were directly fixed and preserved in 96 or 70% ethanol until DNA extraction. DNA extraction was carried out using the GenElute™ Mammalian Genomic DNA Miniprep Kit distributed by Sigma-Aldrich, following the manufacturer's instructions. For molecular identification and phylogenetic reconstruction, we amplified the mitochondrial gene coding for the subunit I of the cytochrome c oxidase (COI) using the universal primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). Polymerase chain reaction (PCR) amplifications were carried out in 20 μL solutions using 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of each primer, 1 U of DreamTaq DNA polymerase (Thermo

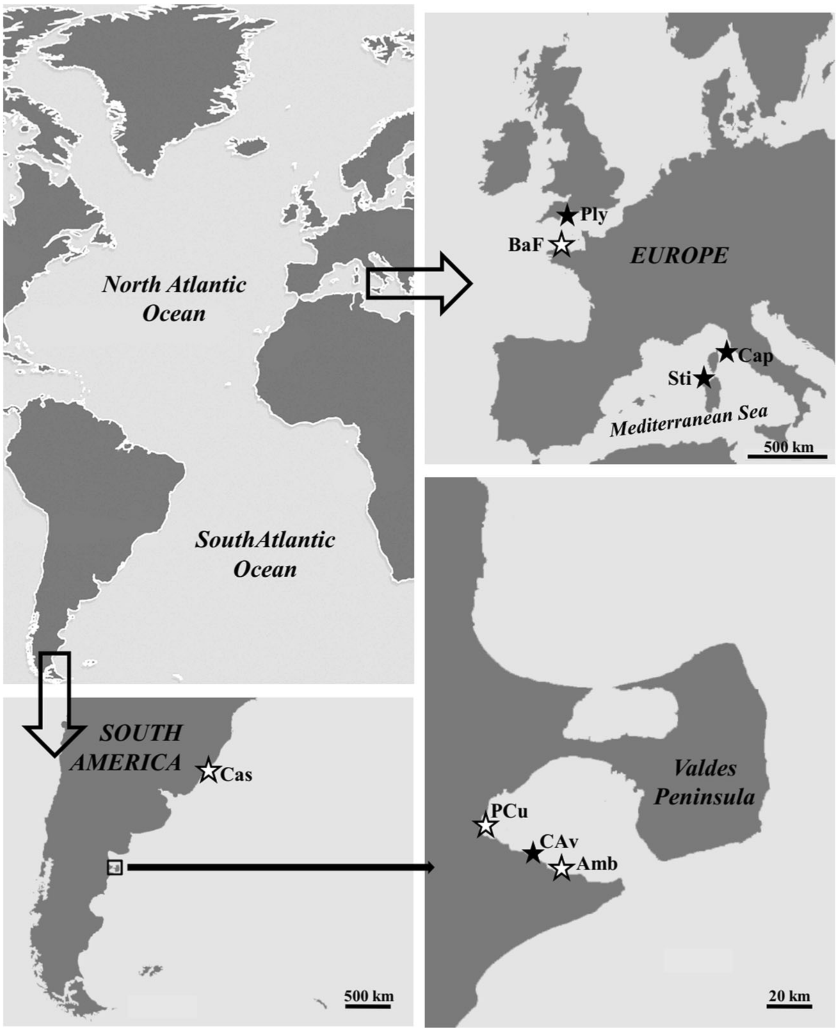


Fig. 1 Sampling localities of *Eulalia clavigera* in western and eastern Atlantic Ocean: Amb Ambrosetti, BaF Beg an Fry, Cap Capraia Island, Cas Cassino, CAv Cerro Avanzado, PCu Punta Cuevas, Ply Plymouth, Sti Stintino. Black stars: populations analysed from the molecular and morphological point of view; white stars: populations analysed from the morphological point of view only

Scientific), and ~ 2.5 ng of template DNA. The PCR profile was set as follows: initial denaturing step at 94 °C for 3 min; 34 cycles of denaturing at 94 °C for 45 s, annealing at 54 °C for 1 min, and extending ay 72 °C for 1 min and a final extending step at 72 °C for 7 min. A negative control was included in each reaction. PCR products were precipitated with sodium acetate and absolute ethanol and sent to Macrogen Europe for sequencing. The obtained sequences were compared with sequences of *E. clavigera* from Banyuls-sur-Mer (Gulf of Lion, Mediterranean Sea) and Plymouth (Northeastern Atlantic, English Channel) retrieved from the GenBank database. Moreover, we employed COI sequences of *Eulalia quadrioculata* Moore, 1906; *Eulalia gracilior* (Chamberlin, 1919), *Eulalia levicornuta* Moore, 1906 and *E. ornata* and *Eulalia viridis* (Linnaeus, 1758) obtained from the GenBank database (Table 1) for phylogenetic reconstruction.

Sequences were aligned with ClustalX 2.1 (Larkin et al. 2007), and alignments were edited in BIOEDIT version 7.2.5 (Hall 1999). Measurement of the genetic differentiation was based on the Kimura-two-Parameter (K2P) model (Kimura 1980). Unrooted Neighbour-Joining (NJ; Saitou and Nei 1987) trees were built using the software MEGA 7 (Kumar et al. 2016), considering reliable nodes supported by a high proportion (> 90%) of replicates in the bootstrap analysis (Felsenstein 1985). The bootstrap test, along with reciprocal monophyly, was used to determine whether a species-like cluster was well supported. The Automatic Barcode Gap Discovery (ABGD) method, generated on the K2P pair-wise distances, was used to support the grouping of the sequences into species. Based on the barcode gap model, this test identifies whenever the average divergence among sequences within species is lower than the average divergence inter-species (Puillandre et al. 2012).

Estimates of *Eulalia* densities were obtained in an abrasion platform located in Cerro Avanzado (42°49.79′S, 64°53.1′W) through the sampling of 50 randomly placed 8 × 8 cm quadrats. The number of specimens in each quadrat was counted using the program ImageJ and divided by the area.

Results

Systematics

Eulalia clavigera (Audouin & Milne-Edwards, 1834) (Figs. 2, 3, and 4)

Phyllodoce clavigera Audouin and Milne-Edwards 1834: 226–228, Pl. 5A, Figs. 9–13

Eulalia clavigera: Bonse et al. 1996: 40–45, Fig. 14 (redescr., syn.); Alós 2004: 193–196, Fig. 69 (SEM photographs)

? *Eulalia viridis*: Morgado and Amaral 1984: 51 (*non* Linnaeus, 1767)

Material examined

Morphology and genetics

South-western Atlantic. **Argentina.** Cerro Avanzado (42°49.79′S, 64°53.1′W) ten individuals (MSNP: P/3892-P/3901), 15 February 2015, coll. T. Vega Fernández and F. Badalamenti. **North-western Atlantic. Great Britain, UK**. Plymouth (50°40.45′N; 4°27.12′W), tide level, rocky shore, five specimens complete, 18 March 2006, coll. F. Pleijel; tide level, rocky shore with holdfast of *Laminaria*, six specimens complete, 21 Mar. 2011, coll. F. Pleijel and A. Nygren. **Mediterranean Sea. Capraia Island, Italy**. Capraia Island (43°1.77′N; 9°50.61′E), 0.5 m, rocky shore with calcareous algae, five specimens (MSNP: P/3024; P/3136; P/3902; P/3903), 18 March 2013, coll. C. Ravaglioli and F. Bulleri. **Northern Sardinia, Italy**. Stintino (40°56.13′N; 8°13′47.86″ E), 0.5 m, rocky shore with calcareous algae, endolithic, five specimens (MSNP: P/3456; P/3904; P/3905; P/3906; P/3907), 15 May 2013, coll. J. Langeneck and M. Casu.

Morphology

South-western Atlantic. **Argentina**. Cerro Avanzado (42°49.79′S, 64°53.1′W), five specimens (CNP INV 993), two complete, others fragmented, 13 August 2007, coll. unknown; 27 specimens; 6 ethanol-preserved specimens

Table 1 GenBank sequences employed in the phylogenetic reconstruction

Species	GenBank accession numbers
Eulalia clavigera (Audouin & Milne-Edwards, 1834)	KT709556.1; KT709559.1; KT709558.1; KT709557.1; KT709568.1
Eulalia gracilior (Chamberlin, 1919)	JQ623500.1
Eulalia levicornuta Moore, 1906	HM473374.1; HM473376.1; HM473375.1; HM473373.1; HM473372.1
Eulalia ornata Saint-Joseph, 1888	KT709567.1; KT709565.1; KT709566.1
Eulalia quadrioculata Moore, 1906	JQ623496.1
Eulalia viridis (Linnaeus, 1767)	KR916829.1; KR916828.1; KR916827.1; KT709563.1; KT709561.1; KT709560.1; KT709564.1

(ECOSUR-OH-P702–706), 2 complete, and 21 formalin-preserved specimens (ECOSUR-P2903), 14 complete, and 7 other anterior fragments, Northern rocky platform, 19 February 2013, coll. E. González, J.M. Orensanz and S.I. Salazar-Vallejo. Punta Cuevas (42°46′S, 64°54′W), Puerto Madryn, nine specimens, six complete, among mussels, 3 December 2009, coll. B. Trovant and J.M. Orensanz. Ambrosetti (42°50.00′S, 64°50.00′W), ten specimens (CNP INV 964), five complete, others fragmented, 15 February 2010, coll. unknown. **Brazil.** Cassino (32°11′S, 52°9′W), five specimens (CNP INV 1647), three complete, others fragmented, 9 March 2008, coll. unknown. **North-eastern Atlantic**. **Bretagne, France**. Beg an Fry, Guimaëc (48°40′04″N, 03°42′27″W), 1 m, rocky shore, two specimens (LACM, formerly SMF 4639, id. *Eulalia viridis* (Linnaeus 1767)), complete, 13 March 1994, coll. D. Fiege.

Description. Complete specimens with up to 168 chaetigers and 78 mm total length, for 3 mm maximum width (smallest 13 mm long, 1 mm wide, 94 chaetigers). Prostomium triangular, almost as long as wide; antennae, palps and tentacular cirri tapered, but tips can be modified due to preservation. Median antenna slightly ahead of eyes, reaching prostomial anterior margin; longest tentacular cirri reach chaetigers 7–8 (6–9 in smaller specimens) (Fig. 3a, d). Pharynx with densely packed papillae, covering its whole surface; 22–30 marginal papillae, with varying shape depending on the degree of its extension (Fig. 3b, e). Dorsal cirri lanceolate, blunt, about twice longer than wide, becoming longer in posterior segments. Dorsal cirrophores narrower than dorsal cirri in anterior chaetigers; progressively wider, as wide as dorsal cirri in posterior chaetigers. Chaetal lobes bilobed, each lobe rounded. Ventral cirri ovate, about twice longer than wide, blunt, usually smaller than chaetal lobes (Fig. 4a, c, f), or as long as them (Fig. 4d–e). Parapodia uniramous, with thick bundles of heterogomph compound chaetae, handle slightly swollen distally, denticulate, hinge teeth blunt, 2–3 times longer than wide, blades tapered, finely denticulate, 6–8 times longer than wide.

Pigmentation, Living specimens are deep green (Fig. 2), including the pharynx; once preserved, the pigment fades off into a greenish hue (Fig. 3e, b). Aged specimens (Fig. 3c–e) turn into brownish or pale brownish, and the pharynx is paler. Darker, glandular spots are present laterally from prostomium, in dorsal cirri, along posterior segmental margins but missing in mid-dorsal regions and basally in parapodial bases. These spots become better defined once the green pigment fades off. Unlike the greenish pigmentation which fades off in aged specimens (Fig. 3), dark brown or blackish, possible glandular spots are present in dorsal cirri, in dorsal cirrophores and in the lower part of parapodial lobes. In dorsal cirri, they can be arranged in irregular series in median chaetigers (Fig. 4b–e), or as an irregular transverse series (Fig. 4f), or not visible (Fig. 4a).

Remarks

Specimens from the south-western and north-eastern Atlantic perfectly match as regards size, colour pattern and morphological features; Mediterranean specimens are often slightly smaller, with yellowish-green (rather than bright green) colour alive, slightly more elongate prostomium and more pointed dorsal cirri. Kato et al. (2001: 387, Table 1) compiled several *Eulalia* species having green pigmentation, but the only one having uniform pigmentation was *Eulalia viridis* (Linnaeus, 1767). Bonse et al. (1996) redescribed *E. viridis* and reinstated *E. clavigera* (Audouin & Milne-Edwards, 1834), but this paper was apparently overlooked by Kato et al. (2001). These two species have slight differences in prostomial, parapodial and pharynx papillation features that allow their distinction. According to Bonse et al. (1996), the length-to-width ratio of dorsal cirri is the most useful character to distinguish between *E. viridis* and *E. clavigera*.

Ehlers (1901) described *Eulalia strigata* from Puerto Madryn, Argentina. He hesitated about the generic placement because his specimen had its pharynx invaginated; he indicated that the body was brownish with a distinct mid-dorsal, longitudinal band, and that the median antenna was placed between the eyes. Since some specimens of *E. clavigera* become darker, sometimes brownish, a comparison with Ehlers' (1901) description is needed. However, the drawing of a parapodium of *E. strigata* (Plate 7, Fig. 18) shows that it is very different from those found in *E. clavigera*: in *E. strigata*, dorsal cirri are oval to rounded, slightly tapered distally, whereas ventral cirri are rounded but markedly longer than the neurochaetal lobe. On the contrary, in *E. clavigera*, dorsal cirri are markedly tapered, and the ventral cirri are oval, slightly pointed and about as long as the neurochaetal lobes. A somehow similar species, *Eulalia magalhaensis* was described by Kinberg (1866, 1910) from Buket Island, Magellan Strait, in shallow subtidal environments. It also has a greenish body and similar prostomial and parapodial features, but dorsal and ventral cirri are lanceolate, acute, not blunt as in *E. clavigera*. All native species of *Eulalia* are therefore clearly different from the introduced *E. clavigera*.

Changes in pigmentation after fixation have been highlighted in the original description. Audouin and Milne-Edwards (1834: 228) indicated that "the overall colour of *Phyllodoce clavigera* is bright green but, through the action of alcohol, changes to metallic brown" ("*La couleur générale de cette Phyllodocé clavigère est d'un vert brilliant qui, par l'action de l'alcool, passe au brun métallique*"). The redescription indicated a homogeneous pigmentation but living animals are paler ventrolaterally. The pharynx distal papillae change their shape depending on the sample treatment. Non-relaxed specimens have globose, low papillae whereas osmotic shocked specimens have them thin, better defined.

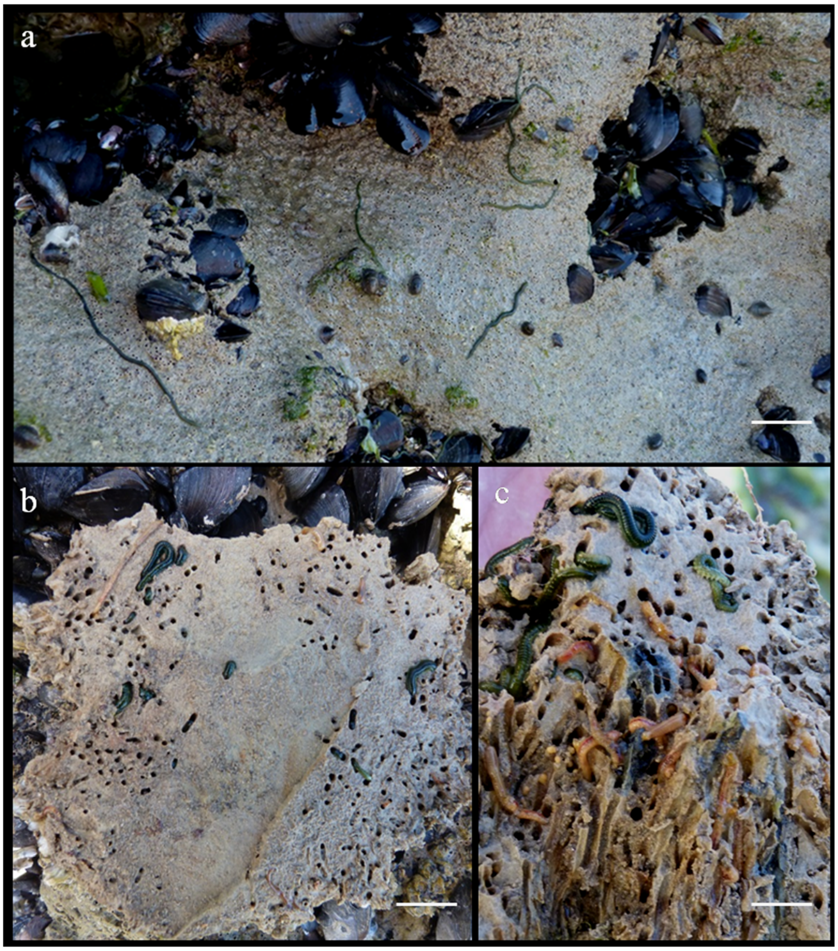


Fig. 2 Live individuals of *Eulalia clavigera* in Cerro Avanzado, Puerto Madryn among *Mytilus edulis* (**a**) and tubes of *Boccardia proboscidea* (**b**, **c**). Scale bars—**a** 2.5 cm, **b** 1 cm, **c** 7.5 mm

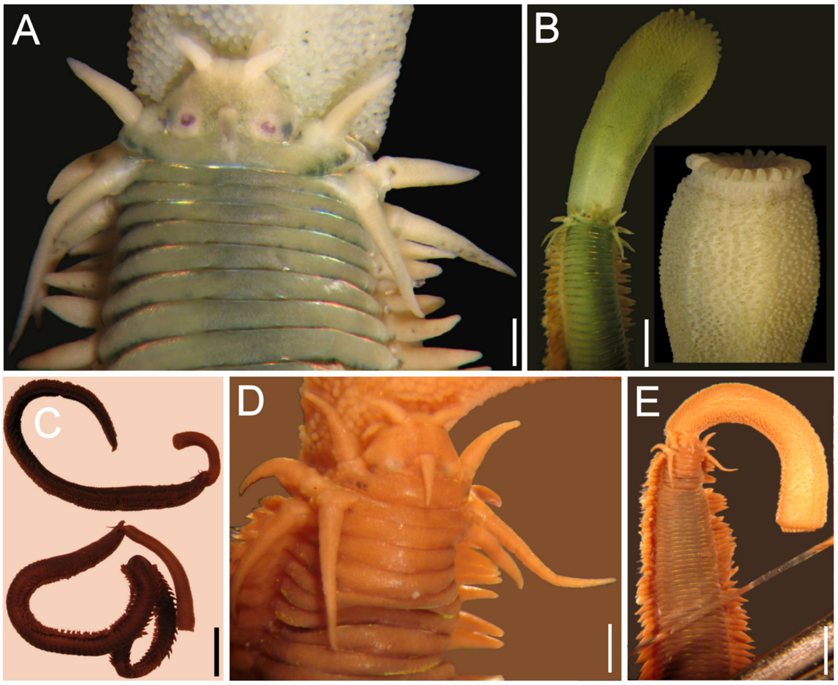


Fig. 3 *Eulalia clavigera* (Audouin & Milne-Edwards, 1834). **a**, **b** Cerro Avanzado, Puerto Madryn, Argentina, freshly preserved specimens (ECOSUR-OH). **c**–**e** Bretagne, France, aged specimens (LACM). **a** Anterior end, dorsal view. **b** Same, showing fully everted pharynx (inset: close-up of pharynx margin). **c** Two complete specimens, one with fully exposed pharynx. **d** Anterior end of above specimen. **e** Same, dorsal view, pharynx exposed. Scale bars—**a** 0.25, **b** 1.4, **c** 3.5, **d** 0.3, **e** 1 mm

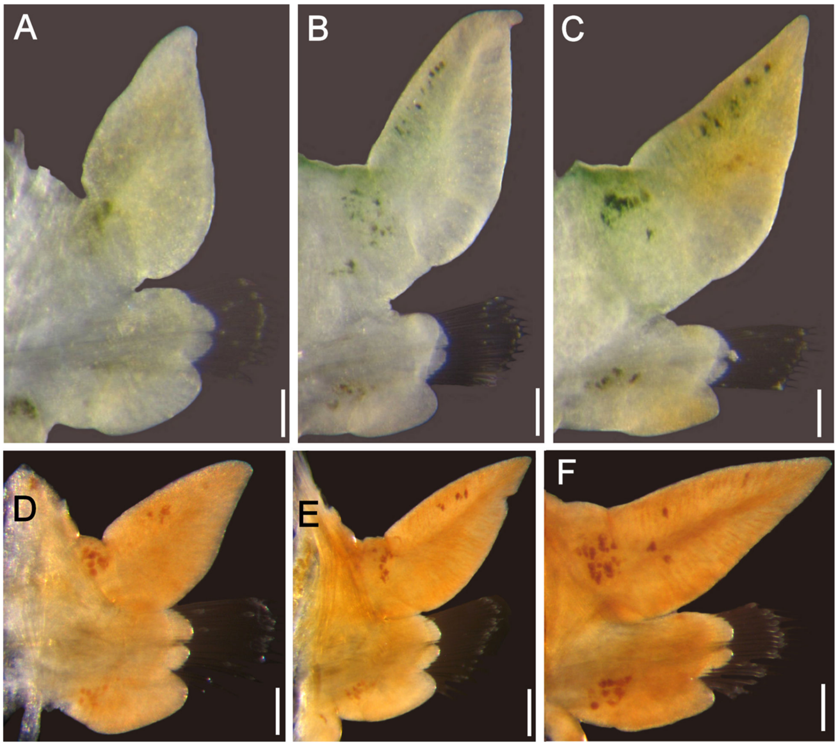


Fig. 4 *Eulalia clavigera* (Audouin & Milne-Edwards, 1834). **a**–**c** Cerro Avanzado, Puerto Madryn, Argentina, freshly preserved specimens (ECOSUR). **d**–**f** Bretagne, France, aged specimens (LACM). **a** Chaetiger 10, right parapodium, posterior view. **b** Chaetiger 50, right parapodium, posterior view. **c** Chaetiger 110, right parapodium, posterior view. **d** Chaetiger 10, right parapodium, posterior view. **e** Chaetiger 50, right parapodium, posterior view. **f** Chaetiger 110, right parapodium, posterior view. Scale bars—**a** 100, **b**, **c** 150, **d** 135, **e** 200, **f** 180 μm

Distribution

The species is naturally present in the UK, France to the Mediterranean Sea (Alós 2004) and southwards to the Canary Islands (Núñez et al. 2005; Núñez et al. 2010). It is now being recorded as an exotic species in Puerto Madryn, Argentina; the examination of southern Brazilian material highlighted the occurrence of individuals morphologically corresponding to this species, even if molecular data are not available. It is likely that individuals identified as *E. viridis* in southern Brazil by Morgado and Amaral (1984) also belong to *E. clavigera*, but we could not study their material. In Argentina, it has been found in intertidal rocky or mixed bottoms, among mussels and barnacles and spionid tube masses (Fig. 2). The mean density recorded on rocky bottoms at Cerro Avanzado was of approximately 90 individuals/m^2, with a maximum density recorded of 468 individuals/m^2.

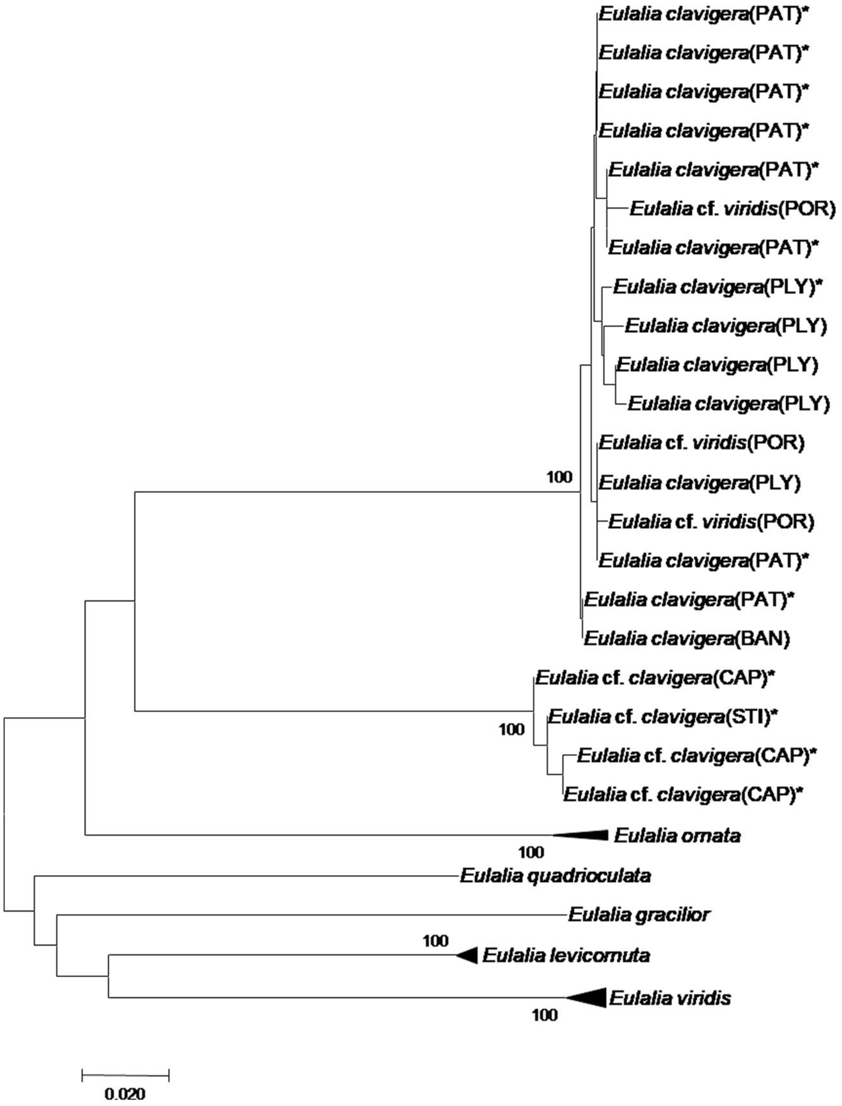
Phylogenetic reconstruction and species delimitation

We obtained for our material 592 bp COI sequences (GenBank accession numbers MG253792 to MG253802). The unrooted NJ tree obtained (Fig. 5) showed that all specimens from Patagonia belonged to a highly supported clade including *Eulalia clavigera* from the north-eastern Atlantic Ocean (PLY) and a single individual from the northern Mediterranean Sea (BAN). These specimens should therefore be identified without any ambiguity as *E. clavigera*. Moreover, individuals from Portugal identified as *Eulalia* cf. *viridis* by Lobo et al. (2016) turned out to belong to the *Eulalia clavigera* clade, and their identification should be changed accordingly. Intraspecific K2P distances detected within this group ranged from the 0 to the 1.2%, and conversely, no trace of geographical differentiation was detected, with specimens from Patagonia, Portugal, Great Britain and the Gulf of Lion showing extremely low distances. By contrast, the remaining Mediterranean specimens examined, originally identified as *E. clavigera*, turned out to be the sister group of the "true" *E. clavigera*, showing similar intraspecific distances (0.2–0.7%) but a high interspecific distance towards *E. clavigera* (19–22%). All other species employed in the phylogenetic reconstruction are clearly distinct from the two lineages, with high interspecific distances (18–28%). The ABGD test univocally identified all divergent lineages as different species, suggesting that *Eulalia* cf. *clavigera* from the Mediterranean Sea actually represents a different, undescribed species.

Discussion

Morphological and molecular characterisation unambiguously demonstrates the occurrence of *Eulalia clavigera* in shallow-water environments of Patagonia. The examined specimens do not show any difference from European material both from the molecular and the morphological point of view; on the other hand, morphological features allow a sure distinction from native *Eulalia* species occurring in

Fig. 5 Tree inferred using the Neighbour-Joining method on Kimura-2-Parameter COI distances. The tree is drawn to scale. Only significant bootstrap values (> 90%) are shown next to the nodes. *herein sequenced. BAN Banyuls-sur-Mer, Mediterranean Sea; CAP Capraia Island, Mediterranean Sea; PAT Puerto Madryn, Patagonia, SW Atlantic Ocean; PLY Plymouth, NE Atlantic Ocean; POR Portugal, NE Atlantic Ocean; STI Stintino, Mediterranean Sea



infralittoral and upper subtidal environments of Patagonia. *E. clavigera* is known to effectively resist dehydration (Kensler 1967), has been recorded from salt-marshes (Nicol 1935) and is often seen crawling among barnacles in European waters (Evans 1949). This ability to thrive in different environments characterised by large variation in chemical and physical parameters could explain why this species has been capable to establish abundant populations in the upper intertidal, among *Spartina* root mats, as it was observed in Puerto Madryn, Argentina. The absence of differentiation between specimens from the north-eastern and south-western Atlantic Ocean strongly supports the hypothesis of a recent introduction of *E. clavigera* in south-western Atlantic waters; the introduction date is currently unknown, but this species has not been recorded in intensive surveys carried out in the 1970s (J. M. Orensanz, *pers. comm.*) and therefore, it is likely that its introduction occurred in the last three decades. The relatively recent development of aluminium industry (1970), that led to a substantial expansion of the small town of Puerto Madryn, and to a significant increase of its international naval connections, is strongly consistent with the hypothesis of a recent unintentional introduction of *E. clavigera* with shipping, either with ballast waters or in fouling communities (Schwindt et al. 2014). On the other hand, specimens from Great Britain, Portugal and northern Mediterranean Sea did not show any trace of genetic differentiation, and intraspecific distances detected were always very low. This suggests a high connectivity among populations of *E. clavigera* and prevents a more precise reconstruction of the origin of the introduced population of *E. clavigera* recorded in the present study.

This study highlights also some uncertainties in the taxonomy of green *Eulalia* species. The distinction at specific level between *E. clavigera* and *E. viridis* was confirmed only recently by Bonse et al. (1996), and Kato et al. (2001) still considered *E. viridis* as the only species with uniform green pigmentation. The distinction between *E. clavigera* and

E. viridis is difficult, especially in environmental monitoring surveys, and the two species are often confused: for instance, *E. viridis* is still reported from the coast of Portugal (Rodrigo et al. 2015), but sequences obtained by Lobo et al. (2016) from Portuguese specimens, and deposited in GenBank as *E. viridis*, actually belong to *E. clavigera* (Fig. 5). Alós (2004) suggested, in agreement with Bonse et al. (1996), that *E. viridis* is a northern boreal and sub-arctic species, and it does not occur in the majority of the European Atlantic coastline, whereas *E. clavigera* is a temperate species, widespread in the Atlantic and in the Mediterranean Sea. The occurrence of *E. clavigera* in the Mediterranean Sea is confirmed by the sequence from Banyuls-sur-Mer in our phylogenetic reconstruction; however, specimens from shallow environments of the Mediterranean Sea sequenced in this work turned out to be only distantly related to both *E. clavigera* and *E. viridis* and should probably be assigned to a currently undescribed species. Possible morphological differences of the new species towards both *E. clavigera* and *E. viridis* are still uncertain and might take into account fine differences in the shape of prostomium and cirri, and in the live colour pattern, that is nonetheless still completely green, without any trace of contrasted drawings. Considering that the Gulf of Lion represents one of the coldest areas in the Mediterranean Sea, showing the occurrence of several Atlantic relict species, it is possible that *E. clavigera* is a relict species in the Mediterranean Sea, whereas the majority of the shallow-water green *Eulalia* in the Mediterranean Sea should be assigned to one, or more, different species. Recent studies on the genus *Eulalia* highlighted that different species are almost impossible to distinguish based on features of the fixed individual, whereas live colour represents one of the most important features in the taxonomy of this genus (Schimmenti et al. 2016). It is very likely, therefore, that further studies will highlight in Mediterranean *Eulalia* cf. *clavigera* a previously unexpected diversity, as already shown for other Phyllodocidae (Nygren and Pleijel 2011), and that the distribution of the "true" *E. clavigera* in Mediterranean environments will turn out to be distinctly narrower.

Taxonomic uncertainties tend to hinder the research about polychaete introductions, and several allegedly alien polychaetes should actually be considered cryptogenic (Çinar 2013). In several cases, new records of polychaete species are merely the consequence of taxonomic revisions (D'Alessandro et al. 2016) or of more detailed studies on poorly known environments (Simboura and Zenetos 2005), and several allegedly alien species have been demonstrated to represent misidentifications of native, often undescribed species (Faulwetter et al. 2008). Molecular identification techniques represent in this frame a powerful tool to disentangle cryptogenic species issues, but until now, their use for polychaetes has been restricted to few taxa, often with implication for human economic activities (Sun et al. 2017), whereas the majority of cryptogenic species cases still remain unsolved. Taxonomic uncertainties most likely prevent also a correct identification of non-native species in the family Phyllodocidae: Çinar (2013) listed only four alien Phyllodocidae, and it is noteworthy that two of them are Lessepsian immigrants (one might be cryptogenic—see Alós 2004) and the remaining two species are currently considered as species complexes, thus of uncertain taxonomy and origin. The complex taxonomy of the genus *Eulalia*, and more specifically of the apparently uniform group of the "green *Eulalia*", could account for the late identification of *E. clavigera* as a new successful invader in Patagonia; however, the combination of morphological and molecular approaches confirmed the identity of these specimens. The present work shows that specimens from Patagonia actually belong to *E. clavigera* and have been most likely introduced in Patagonia from the north-eastern Atlantic Ocean after the 1970s, probably by shipping, even if a more precise origin and path of introduction cannot be traced. The high abundances observed in this species, and its regular observation in intertidal rocky communities, suggest that this species is currently established in the study area. *Eulalia clavigera* is a relatively large predator, feeding mostly on mussels and barnacles (Emson 1977; Rodrigo et al. 2015), even if some studies regard it chiefly as a scavenger of predation remains left by other species (Michel 1970; Morton 2011); the settlement of a large population of this species is therefore expected to largely change trophic interactions within the native assemblage. For this reason, the demography and effect of the introduced *E. clavigera* on native species deserve close monitoring.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Conflict of interest The authors declare that they have no conflict of interest.

Field study Permits and approval of field or observational studies have been obtained by the authors.

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