

# Expansion of lugworms towards southern European habitats and their identification using combined ecological, morphological and genetic approaches

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**ABSTRACT:** This study presents the first record of the recently colonizing lugworm species *Arenicola defodiens* in the Iberian Peninsula, and both *A. defodiens* and *Arenicola marina* in the Ria de Aveiro lagoon (northwest Portugal). We aimed firstly to provide a distinction between these 2 species, using combined morphological and genetic approaches, and secondly to determine their spatial distribution in the lagoon. Morphological characteristics such as annulation pattern, gill morphology, and faecal cast size enabled differentiation between both species, and we provide an updated taxonomic key for the valid species of the Family Arenicolidae known in the Iberian Peninsula. To confirm the distinction between *A. defodiens* and *A. marina*, 16S rDNA and COI gene sequencing analyses were performed. The percentage of nucleotide divergence between *A. defodiens* and *A. marina* was 13.5% for COI and 5% for 16S, and phylogenetic analyses showed that these are closely related species. Recent surveys conducted in Ria de Aveiro lagoon indicate that both species are dispersing throughout the system. Lugworm faecal casts were observed for the first time at 4 sites in 2009, and then at 7 sites in 2011. In 2014, *A. marina* was recorded at 2 intertidal sites of the Ria de Aveiro, which were characterized by very fine and medium sand. *A. defodiens* displayed a wider distribution, being reported at 13 intertidal and 5 subtidal sites where sediment characteristics ranged from mud to medium sand. *A. marina* densities ranged from 20.5 to 26.0 ind. m<sup>2</sup>, higher than those recorded for *A. defodiens*, which did not exceed 3 ind. m<sup>2</sup>. The expansion and the establishment of arenicolids in the Ria de Aveiro lagoon could be a result of the 2009-2010 and 2010-2011 cold winters. Possible consequences of the spread of these bioturbators in this lagunar ecosystem are discussed.

**KEY WORDS:** *Arenicola defodiens* · *Arenicola marina* · Iberian Peninsula · Arenicolidae · 16S · COI

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## INTRODUCTION

Arenicolids, commonly known as lugworms, comprise a group of 4 genera in which about 30 species are recognized worldwide (Hutchings 2000, Rouse & Pleijel 2001). The genera *Arenicola* and *Abarenicola* comprise large worms with a long achaetous posterior region (tail) and include the majority of the species, whereas the genera *Arenicolides* and *Branchio-*

*maldane* are acaudate species, i.e. without a tail (Fauchald 1977).

*Arenicola* species are deposit feeders inhabiting U- or J-shaped burrows in soft marine sediments, and their presence can often be identified by the observation of characteristic castings on soft-bottom intertidal habitats (e.g. Wells 1945). Among the *Arenicola* genus, 3 species are known on European coasts: *Arenicola marina* (Linnaeus, 1758), *Arenicola defodiens*

Cadman & Nelson-Smith, 1993, and *Arenicola cristata* Stimpson, 1856. *A. marina*, commonly known as the blow lug, inhabits estuaries and tidal shores over northwestern European coasts, from the British Isles to the Iberian Peninsula, including Portugal (see Fig. 1A) (Nobre 1903, Rioja 1931, Nobre 1937, Jacobsen 1967, Campoy 1982, Weber 1997, Costello et al. 2001, Bleidorn et al. 2005, Wetthey et al. 2011). *A. defodiens*, also known as the black lug, occurs in subtidal and intertidal habitats of the British Isles, North Sea coasts of Belgium and north of France (Cadman & Nelson-Smith 1993, Müller 2004, Luttikhuisen & Dekker 2010). *A. cristata*, a native species from the Western Atlantic (Bleidorn et al. 2005), is also reported along the coast of the Cantabrian Sea, north of Spain (Costello et al. 2001).

For several years, *A. defodiens* was misidentified as *A. marina* (Cadman & Nelson-Smith, 1990), although a detailed morphological comparison (Cadman & Nelson-Smith, 1993) and genetic information presented by Cadman & Nelson-Smith (1990), based on electrophoresis patterns of 6 enzymes alleles, revealed that they are different species. Recently, Luttikhuisen & Dekker (2010) reported that these 2 species presented 14% of divergence in the mitochondrial DNA fragment COI (cytochrome c oxidase subunit I).

These lugworms constitute key ecological components of intertidal benthic communities in estuarine and coastal habitats (Beukema & De Vlas 1979, De Vlas 1979), and due to their influence as ecosystem engineers, it is important to track the distributions of these species. In northern Europe, these worms are amongst the most economically relevant natural resource, being collected for fishing bait (McLusky et al. 1983). Additionally, several studies have considered the extracellular hemoglobin (HBL Hb) of *A. marina* to be a promising substitute for human blood (e.g. Rousselot et al. 2006, Jernshøj et al. 2013), and its addition to organ preservation solution to provide benefits in organ recovery (Thuillier et al. 2011).

A previous study conducted at the Ria de Aveiro in 1999 reported that no arenicolids were observed throughout the entire lagoon (Rodrigues et al. 2011), which suggests that *A. marina* and *A. defodiens* are not native to the Ria de Aveiro. Therefore, this study presents the first record of *Arenicola defodiens* in the Iberian Peninsula and the occurrence of 2 arenicolid species in the Ria de Aveiro lagoon, northwestern Portugal.

We aimed to (1) distinguish between the 2 species using combined morphological and molecular approaches (analyses of mitochondrial DNA genes:

16S rDNA [16S ribosomal RNA gene] and COI [cytochrome c oxidase subunit I]), (2) determine the spatial distribution and abundance patterns of the *Arenicola* species in the coastal lagoon, (3) determine the possible causes and ecological consequences of the introduction of these species into the ecosystem, and (4) provide a taxonomic key to the Family Arenicolidae from Iberian waters.

## MATERIALS AND METHODS

### Study area and sampling strategy

The Ria de Aveiro is a shallow coastal lagoon located on the northwestern coast of Portugal (Fig. 1), that comprises several narrow channels and extensive intertidal sandflats and mudflats. The lagoon supports highly diverse communities of invertebrates consisting mostly of polychaetes (Rodrigues et al. 2011), some of which are socio-economically important, e.g. polychaetes are exploited for fresh bait, bivalves for human consumption (Cunha et al. 2005, Pires et al. 2012a, Figueira & Freitas 2013).

To establish the diversity, spatial distribution, and to track the distribution of *Arenicola* species in the Ria de Aveiro coastal lagoon, surveys covering the entire system were undertaken in August 2009, December 2011, and between March and April 2014. Some *Arenicola* specimens were also collected during low tide in 2 coastal areas of Viana do Castelo on the northern coast of Portugal (41° 42' N, 8° 51' W), and San Sebastián, northern Spain (43° 19' N, 1° 59' W) in March 2014.

In intertidal areas, the presence of *Arenicola* individuals were detected from their characteristic faecal casts on the sediment surface during low tide. Specimens were collected by digging (~30 cm depth) and were gently removed from the sediment for live observations in the laboratory before being preserved in 96% ethanol. Subtidal specimens were collected using a Ponar grab (sample area = 0.05 m<sup>2</sup>) during March 2014. From all sites, a total of 33 *Arenicola* individuals were collected, preserved in cold 96% ethanol and stored at -20°C in the laboratory to prevent DNA degradation.

To determine arenicolid abundances and their spatial distributions, faecal casts were counted within 10 randomly placed quadrats (0.25 m<sup>2</sup>) at each of the Ria de Aveiro intertidal sites (Volkenborn & Reise, 2006) and collected for determinations of the cast dry weight. At each sampling site, sediment samples were collected with a 5 cm diameter corer for determinations

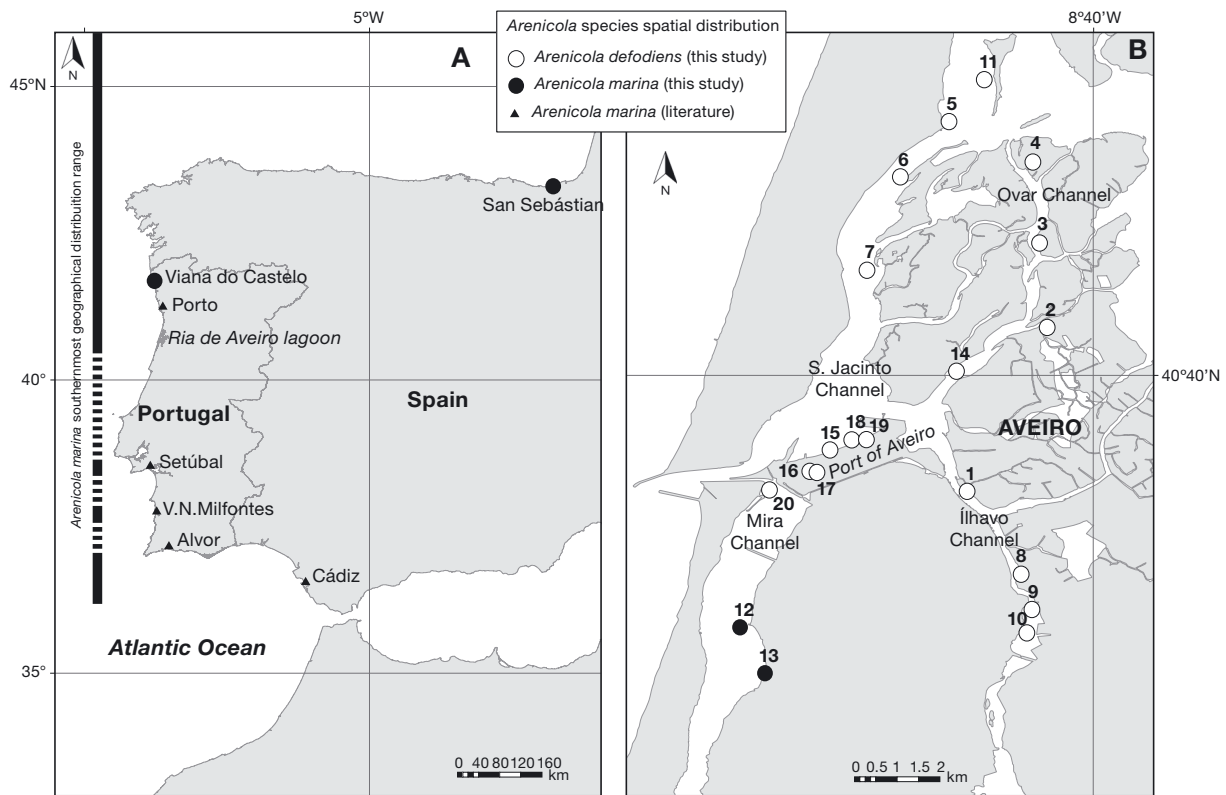


Fig. 1. Distribution of *Arenicola marina* and *Arenicola defodiens* in (A) the Iberian Peninsula, and (B) the Ria de Aveiro lagoon. Black bars indicate the southernmost geographical distribution range of *A. marina*. Black and white dashed bars correspond to unknown distributions of *A. marina*. Numbers correspond to study sites in the Ria de Aveiro (site characteristics are summarized in Table 2)

of sediment grain size and total organic matter. Sediment pH, temperature, redox potential and salinity, were also measured (~5 cm depth) at each site.

### Laboratory procedures

#### Morphological characterization

A total of 60 complete *Arenicola* specimens (fixed with 96% ethanol) were observed under a stereomicroscope. To distinguish between the *Arenicola defodiens* and *Arenicola marina*, the annulation pattern (classification according to Cadman & Nelson-Smith 1993), gill morphology, total segment number, branchial segment number, coloration and the measurement of total length were examined. Two examined specimens of each species were deposited in the Museu Nacional de História Natural, Lisbon (MB29-000341, *A. marina*; MB29-000342, *A. defodiens*) and in the Collection of the Departamento de Biologia, Universidade de Aveiro (DBUA0001714.01, *A. marina*; DBUA0001715.01, *A. defodiens*).

#### Molecular analysis

**DNA extraction.** Total genomic DNAs were extracted with the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions. Purified DNAs were aliquoted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA quality and concentration were tested by 1% agarose gel electrophoresis in 1× TAE buffer, by visual comparison with a DNA ladder mix, and by spectrophotometry at 260 and 280 nm. HWG (high molecular weight) DNAs were subsequently stored at  $-20^{\circ}\text{C}$  for preservation.

**PCR amplification of 16S/COI gene fragments.** Partial regions of the mitochondrial 16S rRNA (~500 bp) and cytochrome c oxidase subunit I (COI) (~700bp) genes were amplified by PCR using the universal primers: 16SarL (5'-CGCCTGTTTATCAAAAACAT-3') and 16SbrH (5'-CCGGTCTGAACTCACATCAGT-3') (Palumbi et al. 1991) for 16S rDNA, and LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Folmer et al. 1994) for COI. PCR reactions

were performed in a final volume of 50  $\mu$ l containing 10–100 ng of genomic DNA, 1  $\mu$ M of each primer, 1 $\times$  PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP (Nzytech) and 0.5 U *Taq* DNA polymerase (Nzytech). Amplification was performed using the following thermal cycling parameters: an initial denaturation at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 1 min, primer-specific annealing at 49°C (for 16S rDNA) or at 45°C (for COI) for 30 s, extension at 72°C for 2 min and final extension at 72°C for 5 min. Amplification products were visualised after agarose gel electrophoresis and ethidium bromide staining to confirm the amplicons' sizes.

**DNA sequencing and analysis.** Nucleotide sequencing of each PCR-amplified fragment (16S/COI) on both orientations and from 2 independent reactions were commercially performed (STAB Vida, Portugal). Obtained sequences were compared with those available in genomic databases using BLAST. Multiple alignments of sense and antisense sequences (from our study and obtained in genomic databases) were conducted using MEGA v6 (Tamura et al. 2013) with ClustalW, using default alignment settings. Then, all sequences were subsequently manually edited by eye using BioEdit Ver. 7.0.0 (Hall 1999). Gap positions and regions that could not be aligned unambiguously were excluded from the analysis. Phylogenetic analysis of the 16S genes was performed by aligning experimental sequences with those of Arenicolidae downloaded from GenBank (see Table 1 for details). Outgroups include *Maldane sarsi* (Malmgren, 1865) (accession number: AY569681) (Family Maldanidae) belonging to the same Order Capitellida, and *Poecilochaetus serpens* (Allen, 1904) (accession number: AY569680) (Order Spionida) belonging to the same Subclass Scolecida. Outgroups were selected on the basis of the phylogenetic study of Arenicolidae by Bleidorn et al. (2005). For COI gene fragments, the experimental sequences were aligned with *A. marina* and *A. defodiens* (the only arenicolid sequences available) downloaded from GenBank (see Table 1 for details). Outgroups included *M. sarsi* of the Family Maldanidae belonging to the same Order Capitellida and *Aricidea nolani* (Webster & Benedict, 1887) (accession number: HQ025025), Order Orbiniida belonging to the same Subclass Scolecida. The phylogenetic analyses of both gene fragments were conducted with the software MEGA v6 (Tamura et al. 2013) by applying Maximum Likelihood and estimating standard error by a bootstrap procedure (1000 replicates).

## Environmental parameters

Sediment grain size was analyzed by wet and dry sieving through a battery of sieves at intervals of one phi ( $\phi = -\log_2$  diameter), following Quintino et al. (1989). The amount of sediment in each sieve was expressed as the percentage of the total sediment dry wt, and was used to calculate the median grain size ( $P_{50}$ ).  $P_{50}$  and the percentage of fines were used to classify the sediment, according to the Wentworth scale: gravel ( $\phi = -2$  to  $-1$ ), very coarse sand ( $\phi = -1$  to  $0$ ), coarse sand ( $\phi = 0$  to  $1$ ), medium sand ( $\phi = 1$  to  $2$ ), fine sand ( $\phi = 2$  to  $3$ ), and very fine sand ( $\phi = 3$  to  $4$ ). Additionally, the final classification adopted the description 'clean' when fines (grain size  $<63 \mu\text{m}$ ) ranged from 0% to 5% of the total dry weighted sediment, 'silty' from 5% to 25%, or 'very silty' from 25% to 50% (Doeglas 1968, Larsonneur 1977). Sediments with  $>50\%$  fines were classified as mud. The total organic matter content was determined by weight loss on ignition at 450°C, according to Byers et al. (1978).

## RESULTS

### Morphological characterization

#### *Arenicola marina*

Total length of the 25 analyzed specimens from the Ria de Aveiro ranged from 10.7 to 14.9 cm ( $11.8 \pm 3.2$  cm, mean  $\pm$  SD). All individuals presented 19 chaetigers, the first 6 abranchiata and the remaining 13 bearing branchiae. All specimens presented the following annulation pattern in the first 4 chaetigers (labelled i–iv): chaetiger i, 2 annuli; chaetiger ii, 3 annuli; chaetiger iii, 4 annuli; chaetiger iv, 4 annuli. Branchiae constituted 8 to 12 stems and lateral branches (3–6) were organized in a dendritic arrangement (Fig. 2A).

The 14 specimens collected in San Sebastián (Spain) and Viana do Castelo (Portugal) showed a similar annulation pattern with the specimens from the Ria de Aveiro (Portugal). Total length ranged from 9.1 to 15.1 cm ( $12.8 \pm 1.8$  cm) and branchiae presented 8 to 11 stems, with a dendritic arrangement.

Live individuals were brown in the first abranchiata chaetigers and pinkish in branchial area. Specimens were found in U-shaped burrows with a conical feeding depression at some distance from the large spiral faecal cast (Fig. 2B); casts had a mean dry wt of  $15.7 \pm 4.8$  g.



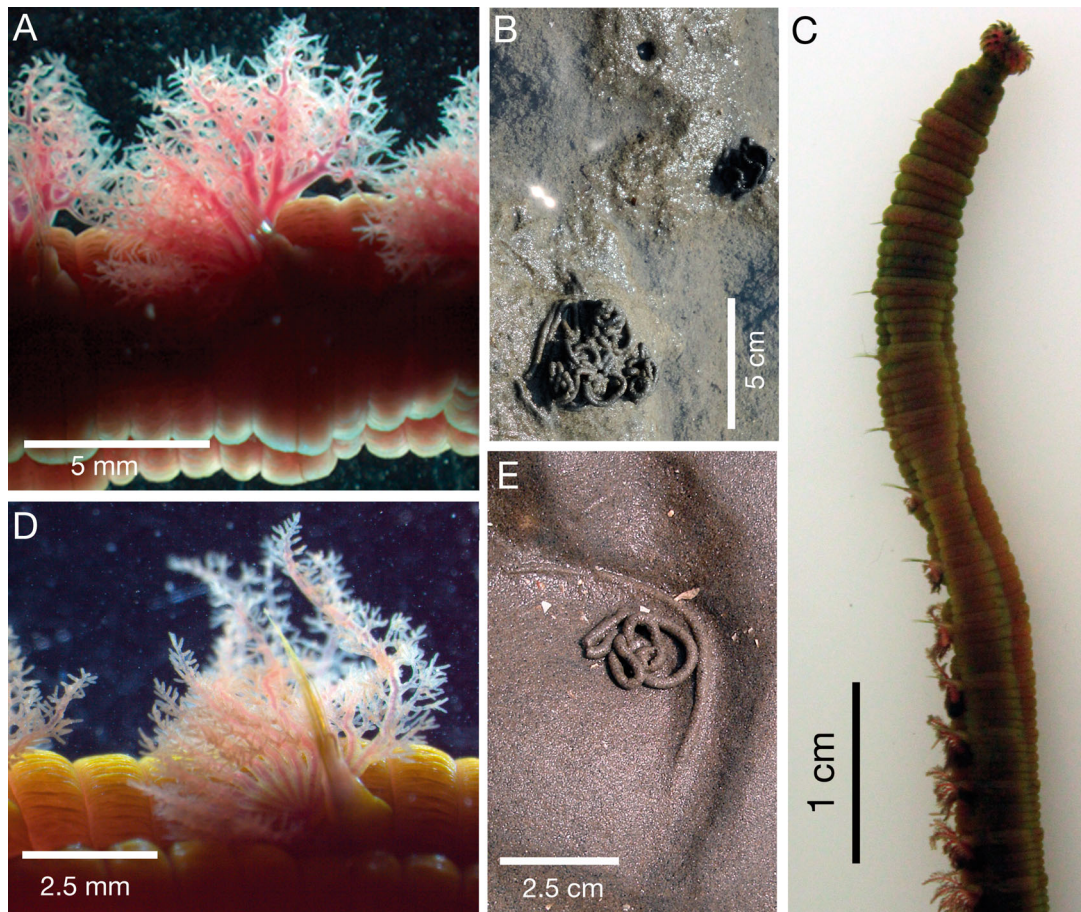


Fig. 2. Arenicolid species morphology and burrow characteristics. *Arenicola marina*: (A) branchiae of a live specimen, (B) faecal casts on sediment surface. *Arenicola defodiens*: (C) anterior end of a live specimen, (D) branchiae of a live specimen, (E) faecal cast on sediment surface

### *Arenicola defodiens*

Total length of the 35 analyzed specimens ranged from 13.3 to 20.2 cm ( $16.6 \pm 3.0$  cm, mean  $\pm$  SE). All individuals featured 19 chaetigers, the first 6 abranchiate and the remaining 13 bearing branchiae. All specimens presented the following annulation in the anterior part: i 2, ii 2, iii 4, iv 4 (Fig. 2C). Branchiae were pinnate with a palmar membrane connecting the branchial stems to the base (Fig. 2D). In the mid-branchial region, the branchiae presented 10 to 14 stems composed of 9 to 12 lateral branches.

Live individuals were greenish along the body, with pinkish branchiae. Specimens were found in U and J-shaped burrows without an obvious feeding depression and with a small spiral-like faecal cast (Fig. 2E); casts had a mean dry wt of  $4.0 \pm 3.0$  g.

### Molecular analysis

A 525-bp 16S fragment and a 702-bp COI fragment were successfully amplified and sequenced from 12 *A. marina* and 21 *A. defodiens* individuals. 16S and COI nucleotide sequences from both species were deposited in the European Molecular Biology Laboratory (EMBL) database, under the accession numbers: KM042095 to KM042101, and JQ950323 to JQ950325.

In the COI gene, 1 substitution was detected for *A. marina* (1 transition of cytosine by thymine at position 389) and 4 substitutions were detected for *A. defodiens*, corresponding to 3 transitions (thymine by cytosine at positions 142 and 248, and guanine by adenine at position 221), as well as one transversion (adenine by cytosine at position 196). As for the 16S gene, all individuals from each species displayed identical nucleotide sequences. Phylogenetic analyses clearly separated *Arenicola* samples collected in

Table 1. 16S rRNA and COI gene sequences used in the phylogenetic analyses in this study (denoted by \*) and in the literature (footnotes a–g). For each haplotype, the species, acronym, aligned sequence length and GenBank accession number are given. n.a. = location not mentioned by authors

Species	Acronym	Sequence length	Genbank accession number 16S	COI	Location	Latitude	Longitude
<i>Arenicola marina</i>	ArMar1	537	KM042095*	–	Ria de Aveiro, Portugal	40° 36' 48.76" N	8° 44' 27.63" W
<i>Arenicola marina</i>	ArMar2	516	HQ691210 <sup>a</sup>	–	n.a.	n.a.	n.a.
<i>Arenicola marina</i>	ArMar3	517	AY577880 <sup>b</sup>	–	n.a.	n.a.	n.a.
<i>Arenicola marina</i>	ArMar4	530	AY523228 <sup>c</sup>	–	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMar5	488	AY340446 <sup>d</sup>	–	Brittany, France	n.a.	n.a.
<i>Arenicola marina</i>	ArMarC1	709	–	KM042097*	Ria de Aveiro, Portugal	40° 36' 48.76" N	8° 44' 27.63" W
<i>Arenicola marina</i>	ArMarC2	709	–	KM042098*	Ria de Aveiro, Portugal	40° 36' 13.29" N	8° 44' 10.31" W
<i>Arenicola marina</i>	ArMarC3	709	–	JQ950326*	Roscoff, France	48° 43' 40.21" N	3° 59' 16.21" W
<i>Arenicola marina</i>	ArMarC4	709	–	JQ950327*	Roscoff, France	48° 43' 40.21" N	3° 59' 16.21" W
<i>Arenicola marina</i>	ArMarC5	654	–	HQ023444 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC6	653	–	HQ023443 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC7	653	–	HQ023441 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC8	652	–	HQ023442 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC9	650	–	HQ023440 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC10	656	–	HQ023440 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC11	646	–	HQ023446 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC12	648	–	HQ023438 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC13	644	–	HQ023439 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC14	628	–	HQ023448 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC15	629	–	GU672158 <sup>e</sup>	Arcachon, France	44° 39' 51" N	1° 09' 38" W
<i>Arenicola marina</i>	ArMarC16	815	–	HQ691225 <sup>a</sup>	n.a.	n.a.	n.a.
<i>Arenicola marina</i>	ArMarC17	660	–	GU672432 <sup>f</sup>	Kandalaksha Bay, Russia	66° 33' 07.2" N	33° 6' 43.2" E
<i>Arenicola marina</i>	ArMarC18	660	–	GU670812 <sup>f</sup>	Kandalaksha Bay, Russia	66° 33' 07.2" N	33° 6' 43.2" E
<i>Arenicola marina</i>	ArMarC19	607	–	HQ023437 <sup>a</sup>	n.a.	n.a.	n.a.
<i>Arenicola marina</i>	ArMarC20	637	–	GQ487319 <sup>g</sup>	Netherlands	n.a.	n.a.
<i>Arenicola marina</i>	ArMarC21	637	–	GQ487320 <sup>g</sup>	Netherlands	n.a.	n.a.
<i>Arenicola marina</i>	ArMarC21	637	–	GQ487321 <sup>g</sup>	Netherlands	n.a.	n.a.
<i>Arenicola defodiens</i>	ArDef1	538	KM042096*	–	Ria de Aveiro, Portugal	40° 40' 36.7" N	8° 40' 34.9" W
<i>Arenicola defodiens</i>	ArDef2	558	JQ950323*	–	Ria de Aveiro, Portugal	40° 41' 20" N	8° 42' 54" W
<i>Arenicola defodiens</i>	ArDefC1	709	–	KM042099*	Ria de Aveiro, Portugal	40° 40' 36.70" N	8° 40' 34.90" W
<i>Arenicola defodiens</i>	ArDefC2	709	–	KM042100*	Ria de Aveiro, Portugal	40° 42' 43" N	8° 40' 39" W
<i>Arenicola defodiens</i>	ArDefC5	709	–	JQ950325*	Ria de Aveiro, Portugal	40° 41' 20" N	8° 42' 54" W
<i>Arenicola defodiens</i>	ArDefC6	637	–	GQ487323 <sup>g</sup>	Netherlands	n.a.	n.a.
<i>Arenicola defodiens</i>	ArDefC7	637	–	GQ487325 <sup>g</sup>	Netherlands	n.a.	n.a.
<i>Arenicola defodiens</i>	ArDefC8	637	–	GQ487324 <sup>g</sup>	Netherlands	n.a.	n.a.
<i>Arenicola defodiens</i>	ArDefC9	637	–	GQ487322 <sup>g</sup>	Netherlands	n.a.	n.a.
<i>Arenicola loveni</i>	ArLov	509	AY569683 <sup>c</sup>	–	Cape Town, South Africa	33° 57' 32" S	18° 27' 40" E
<i>Arenicola cristata</i>	ArCri	526	AY569682 <sup>c</sup>	–	Newport, CAL, USA	33° 36' 39" N	117° 55' 59" W
<i>Abarenicola affinis</i>	AbAff	491	AY569687 <sup>c</sup>	–	Otago Harbour, New Zealand	45° 49' 11" N	170° 38' 06" W
<i>Abarenicola gilchristi</i>	AbGil	512	AY569686 <sup>c</sup>	–	Lamberts Bay, South Africa	32° 05' 54" S	18° 19' 36" E
<i>Abarenicola pacifica</i>	AbPac	483	AY569685 <sup>c</sup>	–	False Bay, Washington, USA	48° 29' 09" N	123° 04' 06" W
<i>Abarenicola clapedredi</i>	AbCla	515	AY569684 <sup>c</sup>	–	False Bay, Washington, USA	48° 29' 09" N	123° 04' 06" W
<i>Arenicolides ecaudata</i>	ArEcca	501	AY569688 <sup>c</sup>	–	Concarneau, France	47° 52' 11" N	3° 55' 21" W
<i>Branchiomaldane vincenti</i>	BrVin	480	AY569690 <sup>c</sup>	–	Concarneau, France	47° 52' 11" N	3° 55' 21" W
<i>Branchiomaldane sp</i>	Brsp	507	AY569689 <sup>c</sup>	–	Morro Bay, CAL, USA	35° 21' 56" N	120° 51' 25" W
<i>Maldane sarsi</i>	MaSar	451	AY569681 <sup>c</sup>	–	Santa Monica Bay, CAL, USA	33° 56' 20" N	118° 30' 16" W
<i>Poecilochaetus serpens</i>	PoeSer	660	–	GU672597 <sup>f</sup>	Kandalaksha Bay, Russia	66° 33' 07.2" N	33° 6' 43.2" E
<i>Aricidea nolani</i>	ArNoC	463	–	–	Arcachon, France	44° 39' 51" N	1° 09' 38" W
		618	–	HQ025025 <sup>f</sup>	Kandalaksha Bay, Russia	66° 33' 07.2" N	33° 6' 43.2" E

<sup>a</sup>Novo et al. (2011); <sup>b</sup>Rouse et al. (2004); <sup>c</sup>Bleidorn (2005); <sup>d</sup>Rousset et al. (2007); <sup>e</sup>Carr et al. (2011); <sup>f</sup>Luttikhuisen & Dekker (2010)

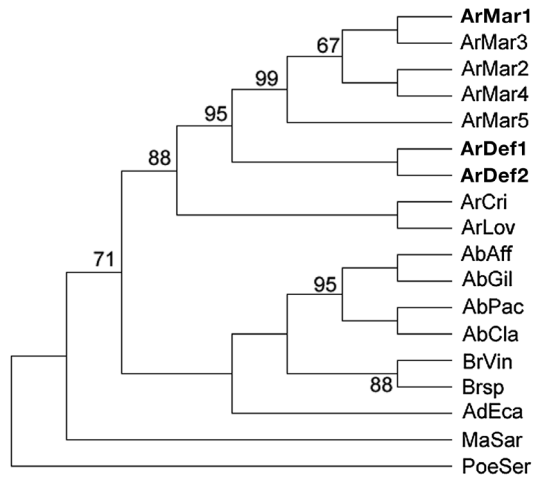


Fig. 3. Maximum Likelihood (ML) tree of 16S rDNA sequences of arenicolid species with bootstrapping values. Sequences from this study are in bold. ArMar = *Arenicola marina*, ArDef = *Arenicola defodiens*, ArLov = *Arenicola loveni*, ArCri = *Arenicola cristata*, AbAff = *Abarenicola affinis*, AbGil = *Abarenicola gilchristi*, AbPac = *Abarenicola pacifica*, AbCla = *Abarenicola claparedi*, AdEca = *Arenicolides ecaudata*, BrVin = *Branchiomaldane vincenti*, Brsp = *Branchiomaldane* sp., MaSar = *Maldane sarsi*, PoeSer = *Poecilochaetus serpens*

the Ria de Aveiro into 2 distinct species, with high bootstrapping values (95/100) (Table 1, Fig. 3). Moreover, 16S phylogenetic analysis showed that *A. marina* and *A. defodiens* are more closely related to each other than to other species of the same genus such as *A. loveni* and *A. cristata* (Fig. 3).

Moreover, deduced amino acid sequence comparison between both species revealed that *A. marina* differed from *A. defodiens* in one amino acid (Alanine to Serine). Maximum Likelihood analysis of COI sequences confirmed the separation of *Arenicola* samples collected in the Ria de Aveiro into 2 distinct species, with high bootstrapping values (99/100) (Fig. 4). The percentage of nucleotide divergence of the 16S and COI genes between *A. marina* and *A. defodiens* was 5% and 13.5%, respectively (nucleotide substitution). The majority of the differences in nucleotides between both species occurred on the third position of the codon and therefore corresponded to silent alterations.

#### Distribution and habitat characterization in the Ria de Aveiro lagoon

Concerning the propagation of *Arenicola* species in the Ria de Aveiro, faecal casts were observed at 4 and 7 sites in 2009 and 2011, respectively, with den-

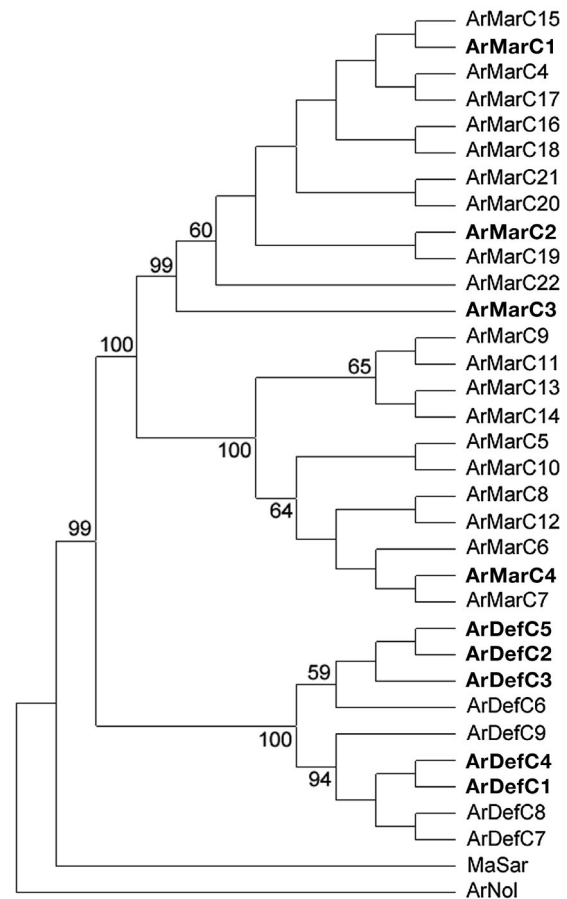


Fig. 4. Maximum Likelihood (ML) tree of COI sequences of arenicolid species with bootstrapping values. Sequences from this study are in bold. ArMarC = *Arenicola marina*, ArDefC = *Arenicola defodiens*, MaSarC = *Maldane sarsi*, ArNoC = *Aricidea nolani*

sities varying from 0.25 to 1 ind. m<sup>2</sup> for *A. defodiens* (6 sites) and 8.75 ind. m<sup>2</sup> for *A. marina* (1 site). In 2014, lugworms were found at 20 localities in the lagoon. *A. marina* was recorded at 2 of these sites located in the Mira channel near oyster cultures, with densities up to 44.0 ind. m<sup>2</sup> (20.5 ± 14.7 ind. m<sup>2</sup>, mean ± SE) at site 12, and 26.0 ± 13.9 ind. m<sup>2</sup> at site 13 (cf. Fig. 1B). This species was found in very fine to medium sands, with the fines ranging between 11.1 and 36.7% and the total organic matter between 0.9 and 2.7%. Salinity of both areas was 28, Eh varied between 16.4 and 80.3, and pH values between 6.85 and 7.40 (see Table 2 for summary of site characteristics).

*A. defodiens* specimens were found at 18 of the 20 study sites, which included 12 intertidal sites, distributed along the Mira, São Jacinto, Ílhavo and Espinheiro channels, and 5 subtidal sites located near the Port of Aveiro at 13 m water depth (cf. Fig. 1B). The

Table 2. Summary of site characteristics in the Ria de Aveiro lagoon. TOM: total organic matter. \* = density reported as ind. per 0.05 m<sup>2</sup> for samples collected with Ponar grab, n.a. = no available data

Site	Habitat	Eh (mV)	Salinity	pH	Fines content (%)	TOM (%)	Sediment type	Density (ind. m <sup>2</sup> ) ± SD			Latitude (°N)	Longitude (°W)	Individuals examined	
								2009	2011	2014				Morphology
<b>A. defodiens</b>														
1	Low Intertidal	44.7	27	7.58	1.06	0.33	Clean fine sand	0	0	1 ± 0.46	40°38'31.32"	8°41'35.58"	2	1
2	Low Intertidal	84.5	34	7.59	6.24	0.66	Silty fine sand	Rare	0.5 ± 0.19	2 ± 0.71	40°40'36.70"	8°40'34.90"	2	2
3	Low Intertidal	287.1	33	7.51	1.33	0.56	Clean fine sand	0	0	1 ± 0.39	40°41'41.71"	8°40'33.73"	2	1
4	Low Intertidal	-230.1	38	7.50	54.63	5.95	Mud	0	0	1 ± 0.44	40°42'43.00"	8°40'39.00"	2	1
5	Low Intertidal	296.2	32	7.31	0.52	0.46	Clean medium sand	0	0.25 ± 0.16	2 ± 0.75	40°43'12.40"	8°41'49.70"	2	2
6	Low Intertidal	232.5	32	7.55	0.72	0.51	Clean fine sand	Rare	1 ± 0.37	2.5 ± 0.76	40°42'30.20"	8°42'26.50"	2	2
7	Low Intertidal	242.2	35	7.66	1.01	0.54	Clean fine sand	Rare	1 ± 0.45	3 ± 0.97	40°41'20.00"	8°42'54.00"	4	3
8	Low Intertidal	14.5	35	6.48	10.30	0.51	Silty fine sand	0	0	2 ± 0.69	40°37'29.00"	8°40'55.00"	2	1
9	Low Intertidal	99.5	28	7.30	22.12	1.91	Silty very fine sand	0	0.25 ± 0.21	1 ± 0.43	40°37'2.00"	8°40'46.00"	2	1
10	Low Intertidal	127.7	28	7.33	2.95	0.58	Clean fine sand	0	0	2 ± 0.54	40°36'45.00"	8°40'51.00"	2	2
11	Low Intertidal	235.2	32	7.40	0.84	0.43	Clean fine sand	0	0	0.5	40°43'44.00"	8°41'22.00"	2	1
14	Low Intertidal	-40.1	33	7.65	18.61	2.7	Silty very fine sand	Rare	0.5 ± 0.27	2.5 ± 0.74	40°40'1.50"	8°41'44.40"	3	2
15	Subtidal (13 m)	-61.3	n.a.	6.25	74.53	9.29	Mud	n.a.	n.a.	1*	40°39'1.97"	8°43'19.49"	1	-
16	Subtidal (13 m)	-87.4	n.a.	7.47	63.01	7.76	Mud	n.a.	n.a.	2*	40°38'47.49"	8°43'34.50"	1	-
17	Subtidal (13 m)	-168.2	n.a.	5.70	82.43	9.31	Mud	n.a.	n.a.	2*	40°38'45.12"	8°43'28.59"	1	-
18	Subtidal (13 m)	-156.6	n.a.	7.32	63.85	8.92	Mud	n.a.	n.a.	1*	40°39'11.36"	8°43'2.94"	1	-
19	Subtidal (13 m)	-170	n.a.	7.22	80.35	9.32	Mud	n.a.	n.a.	2*	40°39'12.19"	8°42'53.45"	2	2
20	Low Intertidal	n.a.	31	7.14	13.12	1.73	Silty medium sand	0	0	2.0 ± 0.78	40°38'26.592"	8°43'58.908"	2	-
<b>A. marina</b>														
12	Medium Intertidal	16.4	28	7.40	36.74	2.64	Very silty very fine sand	0	8.75 ± 3.35	20.5 ± 9.41	40°36'48.76"	8°44'27.63"	11	6
13	Medium Intertidal	80.3	28	6.85	11.14	0.86	Silty medium sand	0	0	26.0 ± 8.45	40°36'13.29"	8°44'10.31"	14	6

density of this species varied between 0.5 and 3.0 ind. m<sup>2</sup> (Table 2). Specimens were found in mud, very fine sand, fine and medium sands, with the fines content ranging from 0.5 to 50.6% and the total organic content from 0.3 to 6.0%. Salinity varied between 27 and 35, pH between 5.70 and 7.66, and Eh between -170.0 and 287.1, according to the wide distribution of the species.

DISCUSSION

This is the first report of *Arenicola defodiens* in the Iberian Peninsula and the occurrence of both *A. defodiens* and *Arenicola marina* in the Ria de Aveiro lagoon, although these species did not co-exist within the same sampling areas. A morphological and genetic comparison was carried out to identify and thus confirm the presence of the 2 *Arenicola* species. We demonstrated the existence of morphological distinctions between both species, such as the annulation pattern of sub-segments at the anterior end and branchiae morphology. These results were corroborated by genetic data, namely by the mitochondrial DNA genes 16S and COI. Despite previous data available on mitochondrial genes for these taxa (Bleidorn et al. 2005, Luttkhuizen & Dekker 2010), this paper presents the first data on 16S sequences for *A. defodiens* and the first coupled phylogeny for both species. Moreover, the Maximum Likelihood analyses among Family Arenicolidae species showed that *A. marina* and *A. defodiens* are closely related when compared with *Arenicola cristata* and *Arenicola loveni*, supporting the morphological similarities observed between both species. In fact, all individuals of *A. marina* and *A. defodiens* presented identical nucleotide sequences for the 16S gene, whilst for the COI gene a 1 point mutation was identified for *A. marina* and 4 for *A. defodiens*. Luttkhuizen & Dekker (2010) also reported that genetic variation was larger in *A. defodiens* rather than in *A. marina*, where 4 haplotypes were described for *A. defodiens*, and 3 for *A. marina*. Compared with the findings of



Cadman & Nelson-Smith (1990), the allozyme variation was higher in *A. defodiens* rather than *A. marina*, where the average gene diversity was 0.23 for *A. defodiens*, and 0.17 for *A. marina* (Luttikhuisen & Dekker 2010). Additionally, these authors suggested that this difference may be the result of population history, where *A. marina* may have suffered more recent or severe recurrent population size bottlenecks.

The mitochondrial genes, 16S rDNA and COI are considered conserved genes, but the relative nucleotide divergence that was obtained between *A. marina* and *A. defodiens* (5% and 13.5%, respectively), is usually observed between different species of polychaetes. In the case of 16S rDNA, within the *Abarenicola* genus the mean divergence between species is 9%, with *Abarenicola pacifica* differing only 4% from *Abarenicola claparedi* (Bleidorn et al. 2005); in the Family Nereididae, the mean sequence divergence between species in the *Hediste* genus is 5.5% (Tosuji & Sato 2012). In the case of COI, sequence divergence for the *Palola* genus had a mean value of 14.5% (Schulze 2006) and 12.5% within the *Hediste* genus (Tosuji & Sato 2010).

In the Ria de Aveiro, *Arenicola* were observed in 4 localities in August 2009, in 7 localities in December 2011, and in 20 localities between March and April 2014. The species *A. defodiens* exhibited a wider distribution in the Ria de Aveiro compared to *A. marina*. However, an inverse pattern was observed regarding the abundance of both species, since *A. marina* was nearly 10-fold more abundant, in agreement with previous studies on European coasts (Flach & Beukema 1994, Cadman 1997, Montserrat et al. 2011, Delefosse et al. 2012). Previous studies in South Wales (United Kingdom) reported the density of *A. marina* reached 25 ind. m<sup>2</sup>, while a maximum of 5 ind. m<sup>2</sup> of *A. defodiens* was found (Cadman 1997). Our knowledge of *A. marina* densities on European coasts is more well-established compared with *A. defodiens*, for which a wide range of density values have been reported. Several studies reported 2- to 3-fold higher densities of *A. marina* compared with the values found for the Ria de Aveiro lagoon, namely in Odense Fjord estuary, Denmark (Delefosse et al. 2012), in Oosterschelde, Netherlands (Montserrat et al. 2011) and in the Wadden Sea, Netherlands (Flach & Beukema 1994). By contrast, much lower densities of *A. marina* were observed in Odense Fjord estuary (1 to 8 ind. m<sup>2</sup>) (Valdemarsen et al. 2011) compared to the present results. Thus, it is likely that both species have well-established populations in the Ria de Aveiro and seem to be dispersing throughout the system.

*A. marina* and *A. defodiens* are not native to the Ria de Aveiro, since benthic community studies have not previously reported these species in this system (Rodrigues et al. 2011), neither on the nearshore, nor the continental shelf off Aveiro (Freitas et al. 2003, Martins et al. 2013, Ravara & Moreira 2013). Small populations of *A. marina* were reported in Viana do Castelo and Oporto and surrounding areas (Nobre 1903, Weber 1997, Wetthey et al. 2011), Setúbal (Nobre 1937), Vila Nova de Mil Fontes (Wetthey et al. 2001), and Alvor (Carpenter 2005) in Portugal (cf. Fig. 1A). Therefore, it is reasonable to assume that *A. marina*, which is widely recognized on northern European coasts, with dense and widespread populations, is increasing its distribution and establishing populations on southwestern European coasts, including Portugal and Spain (e.g. Wetthey et al. 2011). Wasson et al. (2001) reported that 38 exotic invertebrates found in Elkhorn Slough, an estuary in central California, were probably introduced by oyster cultures. Therefore, it is reasonable to assume that *A. marina* was introduced with oyster seeds that came from France, where both species exist, since several oyster cultures at Mira Channel (Ria de Aveiro) receive seeds from France. *A. defodiens* could also be introduced via international shipping traffic (fouling on hulls and ballast water), which was responsible for several introductions (Cohen & Carlton 1995), or travelling on currents. Once introduced, *A. defodiens* larvae may have spread through the system by tidal propagation and currents (Dias et al. 2000), in accordance with the findings of Rodrigues et al. (2011) where marine and transitional communities from the Ria de Aveiro tend to expand more towards the northern channels than to the southern channels.

Previously, *A. defodiens* was only identified on northern European coasts (Cadman & Nelson Smith 1993, Müller 2004, Luttikhuisen & Dekker 2010), with no documented populations between the Somme estuary in North France (Müller 2004) and the Ria de Aveiro. However, sampling effort of Arenicolidae specimens in Viana do Castelo (Portugal) and San Sebastián (Spain) was not sufficient to confirm the absence of *A. defodiens* within this spatial distribution gap. The species *A. defodiens* was described in 1993, previously identified as *A. marina* (Cadman & Nelson-Smith 1993). Therefore, there is the possibility that specimens of *A. marina* from European coasts may have been confused with other species, such as the closely related *A. defodiens*. Similar situations occurred with other species, namely *Diopatra marocensis* which was previously misidentified with *Diopatra neapolitana* on the western coasts of Portu-

gal (Rodrigues et al. 2009), or 4 Lumbrineridae species commonly identified as *Lumbrineris latreilli* or *Hilbigneris gracilis* on the western Iberian continental shelf (Martins et al. 2012).

Climate change induces modifications in ecosystems, namely expansion or retreat of species (e.g. Wethey et al. 2011). Recent studies associated climate anomalies with the expansion of some species in Europe. *Doris biscayensis*, known in Arcachon (SW France) was recently found on the southern Brittany Peninsula (NW France), which was probably enabled by warm summer anomalies (Berke et al. 2010). By contrast, cold-water species, such as *Semibalanus balanoides* were recently reported further south in Europe or showed higher recruitment than in the past. A similar situation occurred with *A. marina* after severe cold winters, namely those during 1962–1963 and 2009–2010, where this species expanded its range throughout the northern coast of France (Wethey et al. 2011). During the winter of 2009–2010, several episodes of severe cold were observed, with mean temperature values decreasing by 1.30°C compared to normal values recorded between 1972–2000 (Instituto Português da Atmosfera e do Ambiente, IPMA 2014). Mean temperature levels of the 2010–2011 winter were also lower, decreasing by 1.19°C compared to the normal values (IPMA, 2014). These winter anomalies may facilitate the expansion and establishment of species in non-native areas, which can explain the occurrence of *A. marina* and *A. defodiens* species in Portuguese coastal systems. Nevertheless, our results demonstrate that *A. marina* and *A. defodiens* continued to spread along the Ria de Aveiro after these cold events, indicating that these species are reproducing in Ria de Aveiro. Watson et al. (1998) reported that the main reproductive period for *A. marina* and *A. defodiens* is during Autumn and Winter, and several experiments with *Arenicola* gametes and/or fertilization occurred at 10 to 12°C (Watson et al. 1998, Campbell et al. 2014). Previous studies reported that the temperature in Ria de Aveiro in winter months (December to February) was 11 to 12°C (Vaz et al. 2005, Pires et al. 2012b); within ranges over which both species can successfully reproduce.

In this study, *A. marina* and *A. defodiens* were found in a wide range of sediment types, from mud to medium sand with organic matter content varying between 0.33 and 9.32%. Cadman (1997) reported that grain size and organic matter were not strong determinants of the presence or absence of either species. Nevertheless, these environmental parameters may have an effect on population density, since Cadman (1997) showed a higher number of *A. marina*

faecal casts in fine sands. Beukema (1976) and Montserrat et al. (2011) demonstrated that *A. marina* preferred sediment with median grain size around 150 µm, inferring that lugworms benefit from sediments that are neither too muddy or sandy. In the Ria de Aveiro lagoon, high densities of *A. defodiens* were found in fine and very fine sand, in agreement with Beukema (1976) and Montserrat et al. (2011). In our study, *A. marina* was observed with densities >20 ind. m<sup>2</sup> at 2 sites comprising different sediment types (very silty, very fine sand and silty medium sand).

*Arenicola* individuals were recorded in intertidal areas, during low tide, by the presence of faecal casts in the sediment surface which are very distinctive for each species. Cadman & Nelson-Smith (1993) noted that *A. marina* tend to produce a conical depression near a large spiral-shaped faecal cast, whereas *A. defodiens* presented a small spiral cast, without an obvious feeding depression. These authors also observed that the mean mass of the faecal casts of *A. defodiens* was 3.48 g (similar to the observations of the present study), and 26.01 g for *A. marina*, almost twice the mass of that observed in our study for this species.

Arenicolids are considered important ecosystem engineers that are capable of mixing both sediment particles and water, thus affecting the biological properties of the sediments (Valdemarsen et al. 2011). Consequently, the regular disturbance of surface sediment modifies the community composition and abundances of other infauna. Previous studies reported that bioirrigation by lugworms facilitate the small zoobenthos by increasing oxygen levels near the ventilated burrows (e.g. Reise 1981, 1983, Lackschewitz & Reise 1998), whilst bioturbation-related disturbances at the sediment surface exclude other organisms, like tube building polychaetes (e.g. *Pygospio elegans*, *Polydora cornuta*, *Spio martinensis* or *Lanice conchilega*) (Volkenborn & Reise 2006) and crustaceans (e.g. *Corophium volutator*) (Flach 1992). Future studies may confirm the ecological interactions between these arenicolids and the tube building polychaetes that are highly abundant in the Ria de Aveiro such as *Diopatra neapolitana*, an important economic natural resource for the local community (Cunha et al. 2005). Although Berke et al. (2010) reported that arenicolids and *Diopatra* species overlap in many worldwide coastal areas, it seems that *Diopatra* spp. and arenicolids may mutually exclude each other; the bioturbator limits *Diopatra* spp. recruitment and *Diopatra* tubes exclude arenicolids.

Additionally, *A. marina* seems to negatively impact the recovery of rooted marine macrophytes, such as

*Zostera marina* (Valdemarsen et al. 2011). In the Mira Channel, *A. marina* was found near *Zostera noltii* meadows. A study carried out in the Odense Fjord estuary by Valdemarsen et al. (2011) suggested that the *Z. marina* recovery was negatively impacted by *A. marina*, even when present in low densities (5–10 ind. m<sup>2</sup>), due to the arenicolid reworking activity that buries the seeds. In this estuary, the lugworm *A. marina* colonized areas previously dominated by *Z. marina* (Valdemarsen et al. 2011). Thus, the spread of *Arenicola* spp. into *Z. noltii* areas in the Ria de Aveiro may be critical, as eelgrass recovery might be severely impaired. The presence of these bioturbators in the Ria de Aveiro complex ecosystem will probably cause changes in the diversity and biomass of vascular plants, infauna and epibenthic fauna. Furthermore, lugworm influences on the irrigation and porewater influx is likely to affect sediment geochemistry (Woodin et al. 1998) and increase microbial activity (Berke et al. 2010).

This study provides information about the main morphological characteristics that allowed us to distinguish *A. marina* and *A. defodiens*. The annulation pattern of the sub-segments was similar for each species in all individuals analyzed. However, previous studies reported some variations in the annulation pattern of both *A. marina* and *A. defodiens* (Cadman & Nelson-Smith 1993, Luttkhuizen & Dekker 2010). Therefore, the annulation pattern should not be used as a single criterion to distinguish either species. Rather, the combined use of annulation pattern, branchiae morphology, faecal cast observation and molecular identification provides a more reliable and accurate species distinction. Future studies of *Arenicola* species population genetics could shed more light on population expansion, and potentially enable us to trace where the population comes from, by analyzing more arenicolids from all areas where they are reported, using universal primers like COI or 16S, or by more specific studies using microsatellites.

Finally, a taxonomic key to the Family Arenicolidae is proposed for the Iberian waters (Appendix 1), which takes into account the morphological observations of the specimens used in the present study, and the recognized species in the Iberian Peninsula based on the studies of Fauvel (1927), Wells (1959, 1963), Fauchald (1977), and de Matos Nogueira & Rizzo (2001).

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**Appendix 1.** Key to Arenicolidae species from the Iberian Peninsula

1. Worms with an achaetous tail... go to (2)  
Worms without a long achaetous tail... go to (6)
2. 19 chaetigers; numerous glandular caeca on the hinder end of the oesophagus; a large, non-retractile prostomium; neuropodia of branchial segments well separated... (*Abarenicola*)... go to (3)  
17–19 chaetigers; a single pair of glandular caeca on the hinder end of the oesophagus; a small, retractile prostomium; neuropodia of branchial segment approach mid-ventrally... (*Arenicola*)... go to (4)
3. First branchiae on chaetiger viii; 6 pairs of nephridia opening from chaetigers iv to ix; neuropodia of chaetigers vi and vii shorter than those of the most anterior chaetigers... *Abarenicola pusilla*\*  
First branchiae on chaetiger vii; 5 pairs of nephridia opening from chaetigers v to ix; first neuropodium approximately as long as those of the posterior branchiate segments; annuli of the first three chaetigerous enlarged and closed... *Abarenicola claparedi*\*
4. 17 chaetigers; 11 pairs of branchiae... *Arenicola cristata*\*  
19 chaetigers; 13 pairs of branchiae... go to (5)
5. Annulation pattern at the anterior end i 2 ii 2 iii 4 iv 4; pinnate gills with a palmar membrane; inhabiting J-shaped deep burrows, without an obvious feeding depression; faecal cast small (less than 5 g) spiral-shaped... *Arenicola defodiens*  
Annulation pattern at the anterior end i 2 ii 3 iii 4 iv 4; dendritic gills, no palmar membrane; inhabiting U-shaped deep burrows with an obvious conical feeding depression at some distance from the large spiral faecal cast (more than 15g)... *Arenicola marina*
6. Relatively large worms; length often exceeding 10 cm; width often exceeding 1 cm; not secreting tubes; branchiae pinnate or bushy present from chaetiger 7 to 17... go to (7)  
Small worms, up to about 2.5 cm long; secreting membranous tubes encrusted with fine sand; branchiae first present from chaetiger 18 or behind, with simple filaments ... (*Branchiomaldane*)... 30 to 50 chaetigers; 17–21 anterior chaetigers without branchiae; 10–28 branchial chaetigers; branchiae with 1–4 simple filaments... *Branchiomaldane vincenti*\*
7. 15–16 anterior chaetigers without branchiae; 13 pairs of nephrids... *Arenicolides ecaudata*\*  
11–12 anterior chaetigers without branchiae; 5 pairs of nephrids... *Arenicolides branchialis*\*

\*Recorded in Iberian waters, but not found in the present study