Universidade de Aveiro Departamento de Biologia
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ADÍLIA DA CONCEIÇÃO MARQUES DE OLIVEIRA PIRES

DIVERSIDADE E BIOLOGIA DE POLIQUETAS DO GÉNERO *DIOPATRA*

DIVERSITY AND BIOLOGY OF POLYCHAETA OF *DIOPATRA* GENUS

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DIVERSIDADE E REPRODUÇÃO DE POLIQUETAS DO GÉNERO *DIOPATRA*

DIVERSITY AND REPRODUCTION OF POLYCHAETA OF *DIOPATRA* GENUS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Ana Maria de Jesus Rodrigues, Professora auxiliar do Departamento de Biologia da Universidade de Aveiro e co-orientação do professor Doutor Franck Gentil, Professor assistente da Estação Biológica de Roscoff, da Universidade Pierre et Marie Curie, Paris 6, França

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

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palavras-chave

Poliquetas, *Diopatra*, Ria de Aveiro, Taxonomia, 16S rDNA, COI, morfologia, filogenia, distribuição, biologia reprodutiva, reprodução, óocitos, desenvolvimento larvar, regeneração anterior e posterior, predação.

resumo

Os anelídeos poliquetas são elementos importantes em ambientes estuarinos e costeiros, pela sua elevada biodiversidade e abundância e pelo papel que têm nas cadeias tróficas. Algumas espécies são intensivamente exploradas para serem utilizadas como isco na pesca desportiva e profissional, como é o caso de *Diopatra neapolitana*. Apesar da importância económica, existem poucos estudos sobre a sua biologia e ecologia. No decorrer deste estudo foram identificadas duas outras espécies do género *Diopatra* em Portugal: *D. marocensis*, inicialmente descrita para a costa de Marrocos e cuja distribuição actual se sabe estender-se a toda a costa Portuguesa e Norte de Espanha e, *D. micrura*, espécie nova para a ciência.

O presente estudo tem como objectivos principais estudar a diversidade e reprodução do género *Diopatra*, bem como a capacidade de regeneração da espécie *D. neapolitana*. Este trabalho aborda a distribuição espacial de *D. marocensis* ao longo da costa Portuguesa e descreve a espécie *D. micrura*, uma nova espécie do género *Diopatra* Audouin and Milne Edwards, 1833. As três espécies coabitam em águas transicionais, onde as espécies *D. micrura* e *D. marocensis* facilmente se confundem com juvenis de *D. neapolitan*a. Foi realizada uma comparação morfológica e genética entre as três espécies.

A espécie *D. neapolitana* coexiste em algumas áreas da Ria de Aveiro com a *D. marocensis*. Apesar destas duas espécies apresentarem padrões reprodutivos muito diferentes, Maio a Agosto é o período principal para a reprodução de ambas as espécies. *D. neapolitana* apresenta um desenvolvimento larvar planctónico, e os óocitos presentes na cavidade celómica são esverdeados e apresentam um diâmetro de 40-240 µm (média = 164.39±40.79 µm) e as fêmeas contêm no celoma milhares de óocitos.

Contrariamente, a espécie *D. marocensis* reproduz-se por desenvolvimento directo no interior do tubo parental. Os óocitos observados no celoma são amarelos com um diâmetro entre 180 e 740 µm (média = 497.65 ± 31.38 µm) e o seu número varia entre 44 e 624 (276.85 ± 161.54). Por seu turno, o número de ovos observados no interior dos tubos varia entre 75 e 298, com um diâmetro entre 600 e 660 µm, e o número de larvas entre 60 e 194.

A proporção machos: fêmeas foi de 1:1 para a população de *D. neapolitana* e entre 1:2 e 1:4 para a população de *D. marocensis*, em que as fêmeas dominam a população durante todo o ano.

O estudo da capacidade de regeneração da espécie *D. neapolitana*, avaliada a partir de experiências de laboratório, revelou que esta espécie é capaz de sobreviver à perda de alguns setígeros. Durante a captura de *D. neapolitana* para vender como isco são normalmente cortados mais de 20 setígeros e de acordo com os nossos resultados a extremidade posterior que fica no tubo não é capaz de regenerar a extremidade anterior; a espécie consegue no entanto recuperar de ataques por predadores.

keywords

Polychaetes, *Diopatra*, Ria of Aveiro, Taxonomy, 16S rDNA, COI, morphology, phylogeny, distribution, life history, reproduction, oocytes, larval development, anterior and posterior regeneration, sublethal predation

abstract

Polychaetes are key elements in the estuarine and coastal food webs, maintaining, beside others, fish and bird populations. Some species are intensively exploited as fresh fish baits for sport fishing such is the case of the *Diopatra* species. In Portugal wild populations of *Diopatra neapolitana* are harvested in various estuaries and lagoons. Very few have been published on the biology and ecology of *Diopatra*, and nothing on the Portuguese populations of these species. During this study it was reported the presence of others two *Diopatra* species in Portuguese waters, *Diopatra marocensis* and *Diopatra micrura* sp. nov.

The aims of this thesis are to study the diversity and reproduction of *Diopatra* populations found in Ria de Aveiro. Also the regenerative capacity of *D. neapolitana* was studied, in order to realize if it contributes to the maintenance of the population, which is intensively exploited to be used as fresh bait.

This study reports the presence of *D. marocensis* in European waters and describes *D. micrura*, a new species of the genus *Diopatra* Audouin and Milne-Edwards, 1833. The three species coexist in transitional waters, where *D. marocensis* and *D. micrura* may be mistaken for young specimens of *D. neapolitana*. A morphologic and genetic comparison between these species was performed.

Besides *D. neapolitana* cohabits with *D. marocensis* in some areas in Ria de Aveiro, both species display very different reproductive patterns, but the main reproduction peak for both species was from May to August. *D. neapolitana* is a broadcast spawning, with free-swimming larvae. The oocytes found in female's body cavity are green with a diameter of 40-240 µm (mean = 164.39±40.79 µm). Otherwise, *D. marocensis* reproduces by direct development in parental tube. The oocytes are yellow and its diameter in females' coelom varied between 180 and 740 µm, with mean 497.65 ± 31.38 µm. The number of oocytes in females' coelom varied from 44 to 624 (276.85 ± 161.54), the number of eggs observed in tubes varied between 75 to 298 and larvae from 60 to 194. The diameter of the eggs found in females' tubes varied between 600 and 660 µm. The male: female sex ratio in *D. neapolitana* was about 1:1 along the year and in *D. marocensis* was between 1:2 and 1:4, with females dominating during all period.

The study of regenerative ability of *D. neapolitana*, evaluated under laboratory conditions, revealed that this species should survive when a few anterior chaetigers are removed, mainly caused by predator attacks. However, the results also suggest that bait digging could impair the survival of the remaining posterior part as usually more than 20 chaetigers are harvested by bait collectors.

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Introduction

1.1. Family Onuphidae

Specimens of the family Onuphidae (order Eunicida), erected as “Onuphiaea” by Kinberg (1865), are usually tubicolous, measuring from a few centimeters to meters, the longest polychaete ever reported, and can be found from the intertidal to the deepest depths in all oceans.

1.2 Genus *Diopatra*

The species of genus *Diopatra* are common in intertidal and shallow subtidal areas of all major oceans although better represented in warmer waters (Paxton, 1986) and includes about 50 species (Budaeva and Fauchald, 2008). In Australia, this genus is represented by seven species (Paxton, 1993), whereas in Europe only *Diopatra neapolitana* Delle Chiaje, 1841 has been recognized until recently. *Diopatra neapolitana* has been reported in intertidal and shallow subtidal habitats, namely in the Red Sea and Indian Ocean (Wehe and Fiege, 2002), the Mediterranean Sea (Gambi and Giangrande, 1986; Arvanitides, 2000; Dagli et al., 2005) and the Atlantic Ocean (Fauvel, 1923; Moreira et al., 2006; Lourido et al., 2008).

Wethey and Woodin (2008) set the northern limit of *D. neapolitana* in France, in Pointe de Penvins (Brittany), and later, Berke et al. (2010) set it only to the French–Spanish border, about 460 km to the south of the previous point, and consider that the species found in northern areas is new to science. Other authors had previously expressed their uncertainty about the cosmopolitan distribution of *D. neapolitana*. Day (1967) noted that several closely related species to *D. neapolitana* had been misidentified and that all records of the species outside the Mediterranean Sea could be considered doubtful. Paxton (1993) also noted that specimens reported by Choe (1960) as *D. neapolitana* could possibly belong to the species *Diopatra sugokai*. On the contrary, *Diopatra aciculata* from Australia is very similar to *D. neapolitana* and Paxton (1993) stated that although she could not observe distinct differences between the two species, retained *D. aciculata* as a separate taxa until more information to the opposite would become available. *Diopatra* taxonomy at the specific level seems still not clear and the need for a

major revision has been recognized (Paxton, 1986), which is in fact a mandatory step prior to the study of species distributional range shifts.

Species of this genus can reach high densities in many habitats (Cunha et al., 2005; Dagli et al., 2005) and play an important ecological role by stabilizing the sediment with their tubes, increasing its structural complexity and potentially enhancing the sediment biodiversity (Bailey-Brock, 1984) while facilitating the settlement and attachment of some algal species (Thomsen and McGlathery, 2005).

D. neapolitana is an important economic natural resource in Ria de Aveiro (Northwestern Portugal) and throughout Europe. The species is intensively harvested to be used as fresh bait. A previous study in Ria de Aveiro, where the present study was undertaken, indicated an annual harvest of 45000 kg, valued at over € 325000 (Cunha et al., 2005). According to Portuguese legislation, bait collection is only allowed by hand gathering or with restricted gear, such as a hoe, operated by licensed personnel (Portuguese legislation: Portaria nº 144/2006). No other legislation exists for the Ria de Aveiro and no management or conservation efforts are currently being developed for this species.

1.2.1. Morphology

The genus *Diopatra* is characterized by the presence of tentacular cirri and spirally arranged branchial filaments, with the prostomium anteriorly rounded to slightly extended (Fig. 1; Paxton, 1986).

The prostomium is composed by 3 antennae and 2 palps (Fig. 1), ceratophores of antennae and palps with 5-20 rings (sometimes with lateral projections), with moderately long to long styles (Paxton, 1986; Paxton, 1998). The frontal lips are subulate and upper lips are oval with distinct distal lobes and median section (Paxton, 1986).

The sensory buds are observed in antennostyles and palpostyles, usually forming 10–26 irregular longitudinal rows (Paxton, 1993).

The nuchal grooves are crescentic, almost circular or rounded. The peristomial cirri are inserted distally on peristomium (Fig 1); lower lip with median section (Paxton, 1986).

The first 3-5 (rarely 7) anterior parapodia (Fig. 1) are modified, being slightly longer than following non-modified. Chaetigers 4-6 with a subulate ventral cirri, having a short transition zone of globular ventral cirri; the dorsal cirri is long to very long. Some species have small ventral lobes on chaetiger 5-25 (Paxton, 1986).

The modified parapodia present uni- to tridentate pseudocompound hooks with short to long hoods and sometimes with 2 rows of minute to small spines in their shafts. The branchiae are present from chaetiger 4-5, being well developed only on anterior part of body (Paxton, 1986, Paxton, 1993).

The pectinate chaetae are observable from chaetiger 5 or later, upper limbate chaetae from chaetiger 1, lower limbate chaetae simple; bidentate hooded subacicular hooks are present usually from chaetiger 15-20 (rarely 12-30) (Paxton, 1986). Pygidium has two pairs of anal cirri, the dorsal usually longer than the ventral pair (Paxton, 1993).

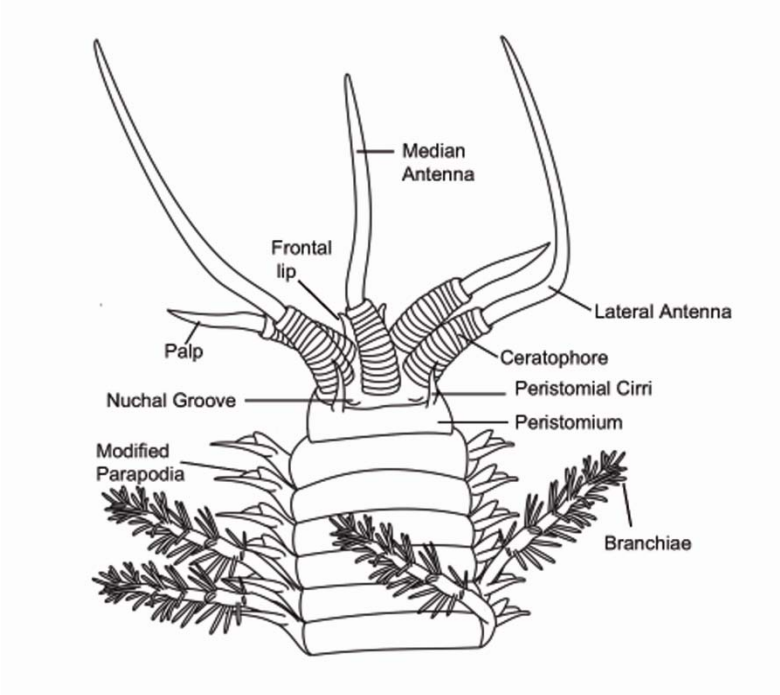

Figure 1 - Morphological characteristics of *Diopatra* species, anterior end, dorsal view.

The tubes of *Diopatra* are cylindrical, robust and consist of a soft inner secreted layer and outer layer of foreign particles, as debris, fragments of sea grass, algae and shells (Paxton, 1986).

Diopatra specimens size is variable, the width of 10th chaetiger varies between 1.3 mm (*D. lilliputiana*) and 13 mm (*D. neapolitana*) (Paxton, 1993; Rodrigues et al., 2009).

1.2.2 Reproduction

Polychaetes are one of the best represented groups in marine benthic communities showing a large variety of feeding types and life strategies. They also are one of the groups with the highest diversity of reproductive traits among marine invertebrates. This is probably due to the relative simplicity of their reproductive system, and to their high plasticity and adaptability to different habitats (Wilson, 1991). Development in Onuphid polychaetes is totally dependent from yolk reserves, with some species having direct development and others lecithotrophy, the individuals feeding only after settlement (Blake, 1975; Giangrande, 1997). The eggs of *Diopatra* species range from 170 to 1400 µm in diameter (Paxton, 1993), and the maximum number that each species could contain ranges from 5 to thousands.

Immature oocytes present nurse cells associated, attached in 2 strings. Nurse cells are common in the Onuphidae family, and they probably transport nutrients taken up from the coelomic fluid to the developing oocytes. Usually larger oocytes had few or no nurse cells attached, probably because nutrients will not be absorbed by the mature oocytes (Blake, 1975).

According to their development type, Paxton (1993) classified *Diopatra* species into four groups: brooding in the parental tube with direct development; direct development in a cocoon; egg masses attached to the parental tube and direct development; and broadcast spawning with a free-swimming stage.

1.2.3 Regenerative capacity

Annelids have the reputation to be able to regenerate. This ability however varies widely within the phylum, and while some species do not have this capacity, others are able to regenerate an entire individual from a single mid-body segment (Bely, 2006). Among the polychaetes, the regeneration ability also differs from species to species. Almost all polychaetes can regenerate appendages, such as palps, tentacles, cirri and parapodia, and most are capable to regenerate the posterior end of the body. Many polychaetes, such as nereids, capitellids and some eunicids, cannot regenerate the anterior part of the body while others have this ability, namely sabellids, syllids, onuphids, maldanids, serpulids, cirratulids and spionids (Brusca and Brusca, 1990; Bely, 2006).

Predation is the mainly cause of injury and subsequently, regeneration among marine benthic invertebrates, but is not the only one. Defensive autotomy, cannibalism, competitive interactions, asexual reproduction, abiotic physical disturbance, and human activities such as bottom trawling are others examples (Lindsay, 2010).

Some *Diopatra* species are capable to regenerate anterior segments, namely some prostomium structures: *D. sugokai*, as *D. amboinensis* (Pflugfelder, 1929), *D. dexiognatha* (Bailey-Brock, 1984; Paxton and Bailey-Brock, 1986), *D. neapolitana* (Bely, 2006; Pires et al., 2011), *D. tuberculantennata* (Budaeva and Fauchald, 2008), *D. cuprea* (Berke et al., 2009), *D. micrura* (Pires et al., 2010) and *D. marocensis* (Pires and co-workers, unpublished data). Paxton and Bailey-Brock (1986) stated that *D. dexiognatha* can readily regenerate anterior and posterior damaged or autotomized regions. Safarik et al. (2006) observed *D. aciculata* individuals regenerating posterior ends. The same authors observed an increase of the regenerating frequency of posterior chaetigers at higher worm densities, pointing out that aggressive encounters among individuals could be density-related.

1.3 Aims

The present work aimed to study the diversity and biology of *Diopatra* species. It includes a description of *Diopatra micrura*, a new species, and the first reference of *D. marocensis* in Portugal and their distribution along the Portuguese coast. Moreover, the reproductive patterns of *D. neapolitana* and *D. marocensis* and the regenerative capacity of *D. neapolitana* were also studied.

Each chapter of this thesis, except the introduction and final remarks, presents different studies with specific objectives and with individual Introduction, Methods, Results and Discussion sections.

The purpose of Chapter 1, Introduction, is to give a brief state-of-the-art overview on the research topic of *Diopatra* genus and to identify the main objectives;

Chapter 2 is dedicated to *Diopatra* diversity and distribution in ria de Aveiro and along the Portuguese coast. It includes a morphological and genetic comparison between the species and a detailed description of *D. micrura*, a new species to the science. The contents of this chapter correspond to two manuscripts published in Estuarine, Coastal and Shelf Science and Zootaxa:

_ Rodrigues AM, **Pires A**, Mendo S, Quintino V (2009). *Diopatra neapolitana* and *Diopatra marocensis* from the Portuguese coast: Morphological and genetic comparison. Estuarine, Coastal and Shelf Science 85: 609–617

_ **Pires A**, Paxton H, Quintino V, Rodrigues AM (2010). *Diopatra* (Annelida: Onuphidae) diversity in European waters with the description of *Diopatra micrura*, new species. Zootaxa 2395: 17–33

Chapter 3 describes the reproductive patterns of *D. neapolitana* and *D. marocensis* based on populations from Ria de Aveiro, and corresponds to two manuscripts, one published in *Marine ecology: an evolutionary perspective* and other submitted to Estuarine Coastal and Shelf Science:

_ **Pires A**, Gentil F, Quintino V and Rodrigues A M (2012) Reproductive biology of *Diopatra neapolitana* (Annelida, Onuphidae) an exploited natural

resource in Ria de Aveiro (Northwestern Portugal). Marine Ecology (http://dx.doi.org/10.1111/j.1439-0485.2011.00463.x).

_ **Pires A**, Gentil F, Quintino V and Rodrigues AM Reproductive patterns in *Diopatra* species: a review with a detailed account of *D. marocensis* (submitted).

Chapter 4 describes the regenerative ability of *D. neapolitana* when submitted to different amputation levels. It corresponds to a manuscript submitted to Estuarine, Coastal and Shelf Science:

_ **Pires A**, Freitas R, Quintino V and Rodrigues AM, Can *Diopatra neapolitana* (Annelida: Onuphidae) regenerate body damage caused by bait digging or predation? Estuarine Coastal and Shelf Science (accepted).

Final remarks are provided in chapter 5 and references in chapter 6.

Chapter 2

Diopatra diversity in Ria de Aveiro, morphological and genetic comparison, with the description of *Diopatra micrura*, new species

2.1 Introduction

Diopatra neapolitana Delle Chiaje, 1841 was until very recently the only recognized species of *Diopatra* in European waters. Recent studies revealed the presence of *Diopatra marocensis* Paxton et al., 1995 in Portugal (Rodrigues et al., 2009) and a species reported as *Diopatra* sp. A from Arcachon to Dunquerque, France, by Berke et al. (2010).

Diopatra marocensis has been recently described by Paxton et al. (1995), from individuals collected off the Moroccan Atlantic coast, where the species dominated a fine sand *Abra alba* community. The species was recorded outside its type locality by Pires et al. (2008), in a number of sites along the Portuguese coast and also by Berke et al. (2010), who mention the species for the lagoon of Óbidos, western coast of Portugal.

D. marocensis occurs sympatrically with *Diopatra neapolitana* in Ria de Aveiro, in the Lagoon of Óbidos and in Villaviciosa estuary (Rodrigues et al., 2009; Arias et al., 2010), but also with *Diopatra micrura* (Pires et al., 2010), in Ria de Aveiro.

In this chapter the main morphological characteristics that allow the distinction between *D. marocensis* and *D. neapolitana* are emphasized. Also the description of a new species, *Diopatra micrura*, **sp. nov**, from several sites of Portugal is included. In order to confirm the distinction between these species, a molecular approach was assessed by characterising two mitochondrial DNA genes, 16S rDNA and COI (cytochrome c oxidase subunit I) (Halanych and Janosik, 2006).

The distribution and habitat of *Diopatra micrura* sp. nov. and *Diopatra marocensis* in Portuguese waters it was also assessed, as well *Diopatra* distribution along Ria de Aveiro.

2.2 Methods

2.2.1 Sampling

On the western coast of Portugal, specimens of *Diopatra* were collected in the Lagoon of Óbidos, "Ria de Aveiro" (intertidal areas) and adjacent shelf area, on the shelf off Nazaré and Guia (off the Tagus Estuary). On the southern coast, specimens were collected near the Guadiana river mouth and in the near shelf off Olhão (Fig. 2). Specimens of *Diopatra sp.* from France (7 from Arcachon and 1 from Marennes Oléron) were kindly sent by Nicholas Lavesque, from Station Marine d'Arcachon.

In Ria de Aveiro, a few intertidal sites were specifically chosen to study the *Diopatra* populations. Here the sediment was collected with a shovel, digging about 30 cm depth, and the *Diopatra* tubes were gently removed from the sediment. Specimens from the other localities were obtained from previous samplings surveys and were re-examined for taxonomic confirmation. In those localities the sediment was collected with grabs, either a 0.1 m^2 Smith-McIntyre (shelf off Aveiro, Guia and Nazaré) or a 0.05 m^2 Ponar (Lagoon of Óbidos and Southern shelf). The samples were washed through a 1 mm mesh sieve and fixed with 4% formalin neutralized with borax. All organisms collected were sorted and identified under a stereomicroscope and then transferred for long-term storage in 70% ethanol.

Sampling surveys in October 2008 and August 2009 were performed in Ria de Aveiro, covering the whole system, to establish the distribution of the *Diopatra* species in this system.

From the Guia shelf and Ria de Aveiro, some specimens of *D. marocensis* and *D. micrura* were also collected for genetic studies. The Guia specimens were preserved in ethanol (96%) and those from Ria de Aveiro were kept cold during field sampling and frozen at the laboratory (−20 C). For the same purpose, individuals of *D. neapolitana* were sampled in Ria de Aveiro and handled as *D. marocensis* and *D. micrura* specimens collected in this system. Also two *Diopatra* sp. specimens from Arcachon Bay, France, preserved in ethanol (96%) were also used, for genetic studies.

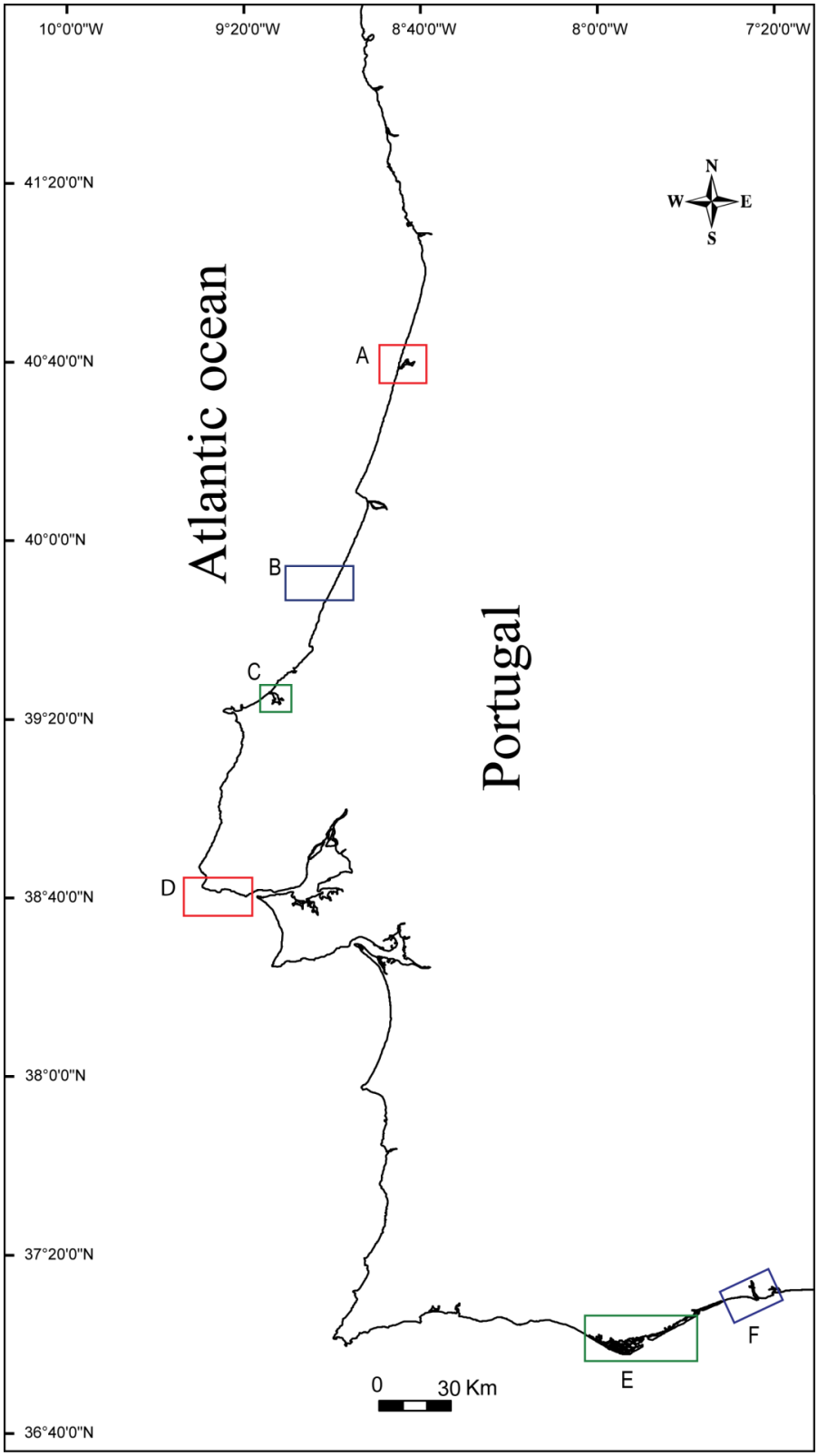

Figure 2 - Sampling areas where *D. micrura* **sp. nov.** and *D. marocensis* were found (red - presence of both species; blue – *D. micrura;* green – *D. marocensis*): A - Aveiro (shelf and Ria); B - coastal shelf off Nazaré; C – Lagoon of Óbidos; D - Guia, coastal shelf off Tagus estuary; E – shelf off Olhão F - coastal shelf off Guadiana estuary.

In order to describe the sediment environment, an additional sample was collected from each site. Samples for grain size analysis were stored in plastic containers and for the total volatile solids analysis (organic matter) samples were stored in a cold environment and frozen at −20 ºC in the laboratory.

2.2.2. Laboratory procedures

2.2.2.1. Morphological characterization and data analysis

In the laboratory, 602 specimens of *Diopatra marocensis* (78 from Ria de Aveiro, 50 from the Lagoon of Óbidos, 474 from Guia), 243 specimens of *Diopatra neapolitana* from Ria de Aveiro, 88 specimens of *D. micrura* (5 from Ria de Aveiro, 9 from the shelf off Aveiro, 3 from the shelf off Nazaré and 71 from Guia and 8 specimens of *Diopatra sp*. from France (7 from Arcachon Bay and one from Marennes Oléron, about 150 km North of Arcachon) were examined for morphological studies.

The individuals were identified and measured, for total length in complete specimens, and for the width of chaetiger 10 (without parapodia). The numbers of chaetigers in complete specimens, rings in the ceratophores, whorls of the branchia, teeth in the pectinate chaeta and the first chaetiger with subacicular hooks were recorded. The last chaetiger with branchiae was registered. The colour pattern and the form of the prostomium of the two species were described, based upon the observation of live specimens. Fixed specimens of *D. micrura* were also measured for the length of antennae and palps. The colour pattern and the form of the prostomium of the species were described also in preserved specimens. The terminology used for the prostomial appendages followed Paxton (1998). The relationship between the width of the 10^{th} chaetiger, taken as a measure of the specimen size, and other morphological descriptors was analysed through linear regression analysis.

Using the data recorded for the width of the 10^{th} chaetiger, the number of rings in the ceratophores, the number of whorls in the branchiae, the number of teeth in the pectinate chaetae, the first chaetiger to show subacicular hooks and

the presence-absence of ventral lobe in the parapodia 5–20, a data matrix was constructed using as many specimens per species as possible (41 *D. micrura* **sp. nov.**, 35 *D. neapolitana*, 35 *D. marocensis* and 8 *Diopatra* sp. specimens obtained in France). Following normalisation of the variables, the morphological data matrix was submitted to classification analysis, using Un-weighted Pair Group Mean Average upon the Euclidean distance matrix between specimens, and ordination, using Principal Components Analysis, with the software PRIMER v6 (Clarke & Gorley, 2006).

The holotype and five paratypes of the new species, *D. micrura*, were deposited in the Museu Nacional de História Natural, Lisbon (MNHN), five paratypes in the Museo Nacional de Ciencias Naturales, Madrid (MNCNM), and six paratypes in the Australian Museum, Sydney (AM). The remaining specimens (including specimens used for DNA sequencing) are kept at the Departamento de Biologia, Universidade de Aveiro.

A more detailed morphological study of *D. neapolitana*, *D. marocensis* and *D. micrura* was based on scanning electron microscopy (SEM). Specimens stored in 70% ethanol were dehydrated in graded ethanol series and critical point dried in a Bal-Tec CPD-030 critical point dryer, using ethanol as a transition fluid. After drying, specimens were sputter coated with gold: palladium alloy 60:40 in a Polaron sputter coating system. SEM micrographs of *D. neapolitana* and *D. marocensis* were taken in a JEOL JSM-5400 scanning microscope. SEM micrographs of *D. micrura* were taken in a Hitachi SU-70 scanning microscope.

2.2.2.2. Genetic characterisation and data analysis

DNA extraction

Specimens of *Diopatra marocensis* collected in the Guia area (15 specimens) and Ria de Aveiro (30 specimens), specimens of *D. neapolitana* collected in Ria de Aveiro (45 specimens) and specimens of *D. micrura* collected in Ria de Aveiro, were used for the genetic analyses. Total genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions.

Purified DNA was aliquoted in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 ºC, until required.

PCR amplification of 16S/COI genes

Partial regions of the mitochondrial 16S rDNA (~500 bp) and cytochrome c oxidase subunit I (COI) (~700 bp) genes were amplified by PCR using the following primers: 16S rDNA: 16SarL (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SbrH (5′-CCGGTCTGAACTCACATCACGT-3′) (Palumbi et al., 1991); COI: LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994).

PCR reactions were performed in a final volume of 50 µl containing 10–100 ng of genomic DNA, 1 µM of each primer, 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP (Promega) and 0.5 U Taq DNA polymerase (Promega). Amplification occurred on a MJ Mini Thermal-Cycler (Citomed) with the following thermal cycling parameters: initial denaturation at 94 ºC, 3 min, followed by 34 cycles of: denaturation at 94 ºC, 1 min; primer-specific annealing 49 ºC (16S rDNA) or 45 ºC (COI), 30 s; extension at 72 ºC, 2 min and final extension at 72 ºC for 5 min.

Amplification products were visualised, after agarose gel electrophoresis and ethidium bromide staining, to confirm the sizes of the amplicons.

DNA sequencing and analysis

Nucleotide sequencing of each PCR-amplified fragment (16S/COI) on both orientations and from two independent reactions were commercially performed (STAB Vida, Portugal).

Sequences were analysed using the Biological Sequence Alignment Editor BioEdit version 7.0.0 (free software by Tom Hall, Department of Microbiolgy, North Carolina State University). Genetyx-WIN Version 5.1 (Software Development, Tokyo) was employed to determine the divergence percentage between the

various *Diopatra* species, for both genes and also compared with others sequences deposited in the EMBL database for the species *D. aciculata*.

These sequences were analysed together in a single data set, separately for each gene, with *Marphysa sanguinea* (Montagu, 1813) as the outgroup. The data set sequences were aligned in MEGA v4 (Tamura et al., 2007) with CLUSTALW, using the default alignment settings.

The phylogenetic analysis were conducted with the computer program MEGA v4 (Tamura et al., 2007) by applying Neighbor Joining (NJ). To verify the robustness of the internal nodes of NJ trees, bootstrap analysis was carried out using 1,000 pseudo replicates.

2.2.2.3. Grain-size and Total Volatile Solids analyses

Sediment grain size from Ria de Aveiro was analysed by wet and dry sieving following Quintino et al. (1989) and sediment was characterized regarding the grain size classes: gravel (particles with diameter above 2 mm), sand (0.063–2.000 mm) and fines content (<0.063 mm). The amount of sediment in each grain size class was expressed as a percentage of the whole sediment, dry weight. The data were used to calculate the median value, P50, expressed in phi ($\phi = -\log^2 mm$) units, corresponding to the diameter that has half the grains (dry weight) finer and half coarser. Given that no detailed grain size analysis was performed for the fines fraction, the median could not be calculated for the samples with more than 50% fines content. These sediment samples were classified as mud. Sands were classified using the median, expressed in ϕunits, according to the Wentworth scale (Doeglas, 1968): very fine sand (median between 3 and 4 ϕ); fine sand (2–3 ϕ); medium sand (1–2 ϕ) or coarse sand (0–1 ϕ). The final classification adopted the description “clean“, “silty” or “very silty”, when fines were ranging from 0% to 5%, from 5% to 25%, and from 25% to 50%, respectively, of the total sediment, dry weight (Quintino et al., 1989).

Total volatile solids (organic matter), from Ria de Aveiro, were determined as weight loss on ignition at 450 C during 5 h (Byers et al., 1978 and Kristensen and Anderson, 1987) of 1 g sediment sample after an initial drying at 60 C for 24 h.

The grain-size data and total volatile solids from the others localities were obtained from anterior works: shelf off Aveiro from Freitas et al. (2003); Guia, off the Tagus Estuary from Sampaio et al. (2010); Southern coast from Freitas et al. (2011) and the data from shelf off Nazaré is unpublished.

2.3 Results

2.3.1 Morphological comparison between *Diopatra marocensis* and *Diopatra neapolitana*

The terminology used in this work to describe the morphology of both species followed Paxton (1998) and is presented in Fig. 3.

The main features to discriminate *Diopatra neapolitana* from *Diopatra marocensis* are summarized in Table 1 which also includes the characteristics referred to by Paxton et al. (1995) for *D. marocensis* and Fauvel (1923) and Dagli et al. (2005) for *D. neapolitana*.

In their adult stage, the two species present different sizes, with *Diopatra marocensis* being smaller than *Diopatra neapolitana* both in terms of body length and width and also with fewer numbers of chaetigers (cf. Table 1).

The colour pattern, observed in live individuals, varied in both species but a general pattern can be described: *Diopatra marocensis* presented a pinkish colour (Fig. 3B), with more whitish parapodia. The prostomium and the ceratophores showed a brown pigmentation, with the area of the nuchal grooves more whitish. The frontal lips were also whitish but brown pigmented at the base. Along the segments, both species presented a brown mid-dorsal patch, in our specimens (Fig. 3C and D) forming a line along the medium dorsal anterior part of the body, up to chaetigers 15–20 in *D. marocensis*, (width: 0.5–1 mm), and chaetiger 30–40 in *Diopatra neapolitana* (width: 1–1.4 mm). Almost all individuals presented many white irregular small spots on the anterior end, dorsal view (Fig. 3B). In *D. neapolitana* such white spots are more evident on the antennae and palps (Fig. 3D). However the white spots are not visible in preserved specimens. The overall colour in *D. neapolitana* is iridescent greenish (Fig. 3D). In the males, the body

area with gametes acquires a cream colour in the reproductive period. The frontal lips of *D. neapolitana* were brown from the base up to the middle of their length (Fig. 3A). Brown rings in the ceratophores were clear on the antennae and palps (Fig. 3A and D). The nuchal grooves were greenish and lighter than the prostomium. Up to the first six chaetigers the overall colour is of a dark brown, becoming lighter and greenish in the rest of the body.

Table 1 - Morphological characteristics used to distinguish *D. neapolitana* from *D. marocensis*. The values given for the present study correspond to the mean ± standard deviation, with the range between brackets (n = 35 for each species).

	Present study		Paxton et al. (1995)	Fauvel (1923)	Dagli et al. (2005)
Character	*D. marocensis*	*D. neapolitana*	*D. marocensis*	*D. neapolitana*	
Length (cm)	8.93 ± 1.98	36.39 ± 13.50	3.5	15–50	34.7
Width of 10th chaetiger (mm)	2.97 ±0.66	7.08 ±1.68	2.0±4.5	-	7.74
Number of chaetigers	141.69 ± 22.80	269.20 ± 31.16	112	200–300	239
Colour	Pinkish	Greenish iridescent	Pale	Greenish iridescent	Brownish
Number of rings of the ceratophores	8.54 ± 0.82 (6–9)	15.40 ± 0.50 (15–16)	7–9	–	15–16
Nuchal grooves	Crescentic	Rounded	Crescentic	–	Sub-triangular
Chaetigers where branchiae begin	4.14 ± 0.36 (4–5)	4.46 ±0.51 (4–5)	4–5	4–5	-
Maximum number of branchial whorls	7.80 ± 1.13 (6–9)	16.20 ± 1.18 (14–18)	6–9	-	14
Chaetiger where branchiae finish	33.77 ± 2.96 (26-38)	64.40 ± 3.53 (56–70)	30–41	60–70	65
Limbate serration of the chaetae	On shelf	All border line	On shelf	-	Coarsely serrated
Nr of teeth of the pectinate chaetae	14.69 ± 2.01 (11-20)	6.60 ± 1.33 (5-10)	11-20	6-9	6-10

The prostomium was rounded anteriorly in *Diopatra marocensis* and slightly pointed in *Diopatra neapolitana*. The ceratophores of *D. marocensis* antennae and palps presented 6–8 proximal rings and 14–15 rings in *D. neapolitana*, with a longer distal ring in both species (Figs. 3B and D; 4A; 5A; cf. Table 1). The antennae and palps, with interrupted longitudinal rows of sensory papillae, 16–18 in the case of *D. marocensis* (Fig. 4B and C) and 20–22 for *D. neapolitana* have been observed at SEM. The nuchal grooves were crescentic in *D. marocensis* and rounded in *D. neapolitana* (Fig. 3, Fig. 4 and Fig. 5) and the peristomial cirri were about twice the length of the peristomium in both species. Four larger modified parapodia (in the first four chaetigers), with rounded prechaetal and subulate postchaetal lobes were observed in both species (Figs. 4F; 5D). The prechaetal lobes were observed up to chaetigers 6–10 in *Diopatra marocensis* and up to 15–20 in *Diopatra neapolitana* (Fig. 5E).

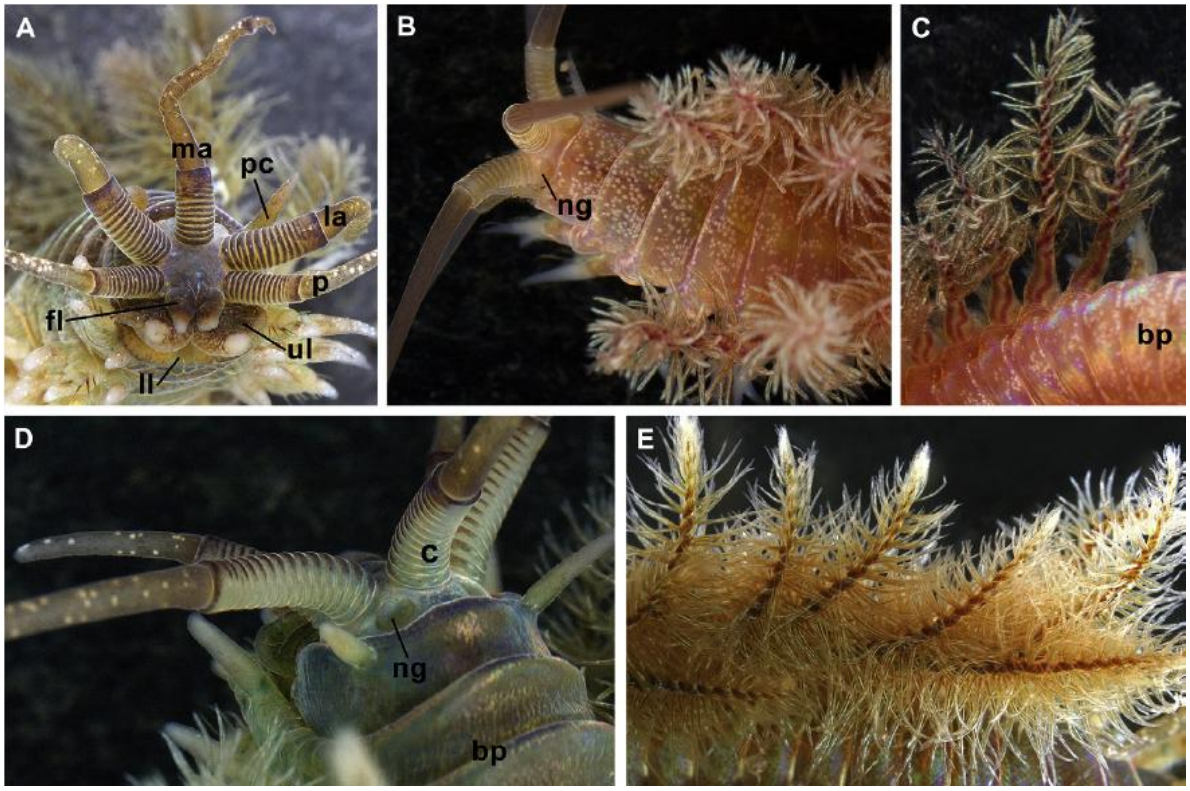

Figure 3 - Morphological characteristics of live specimens of *Diopatra marocensis* and *D. neapolitana*: A – prostomial and peristomial appendages of *D. neapolitana* (sensu Paxton, 1998); B – anterior end of *D. marocensis*, dorsal view; C – branchiae of *D. marocensis*; D – anterior end of *D. neapolitana*, dorsal view; E – branchiae of *D. neapolitana*. bp – brown patch; c – ceratophores; fl – frontal lip; la – lateral antenna; ll – lower lip, ma – median antenna; ng – nuchal groove; p – palp; pc – peristomial cirrus; ul – upper lip.

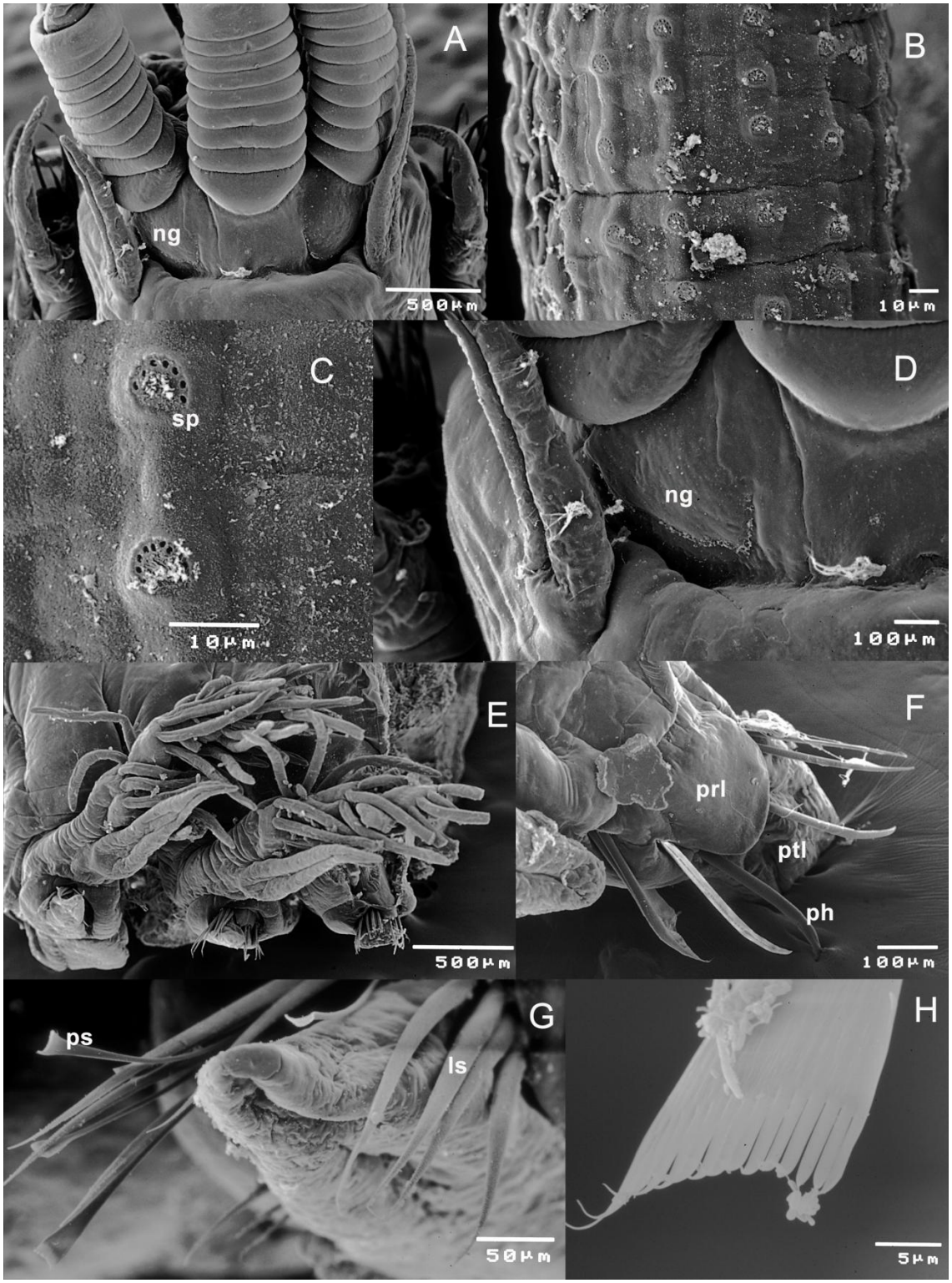

Figure 4 - Scanning electron micrographs of *Diopatra marocensis*: A – prostomium, dorsal view; B – rows of sensory papillae on antenna; C – enlarged sensory papillae of antenna; D – nuchal groove; E – branchiae; F – modified parapodium; G – parapodium of chaetiger 6; H – chaetiger pectinate chaeta. ls – limbate chaeta; ng – nuchal groove; ph – pseudocompound hook; prl – prechaetal lobe; ps – pectinate chaeta; ptl – postchaetal lobe; sp – sensory papilla.

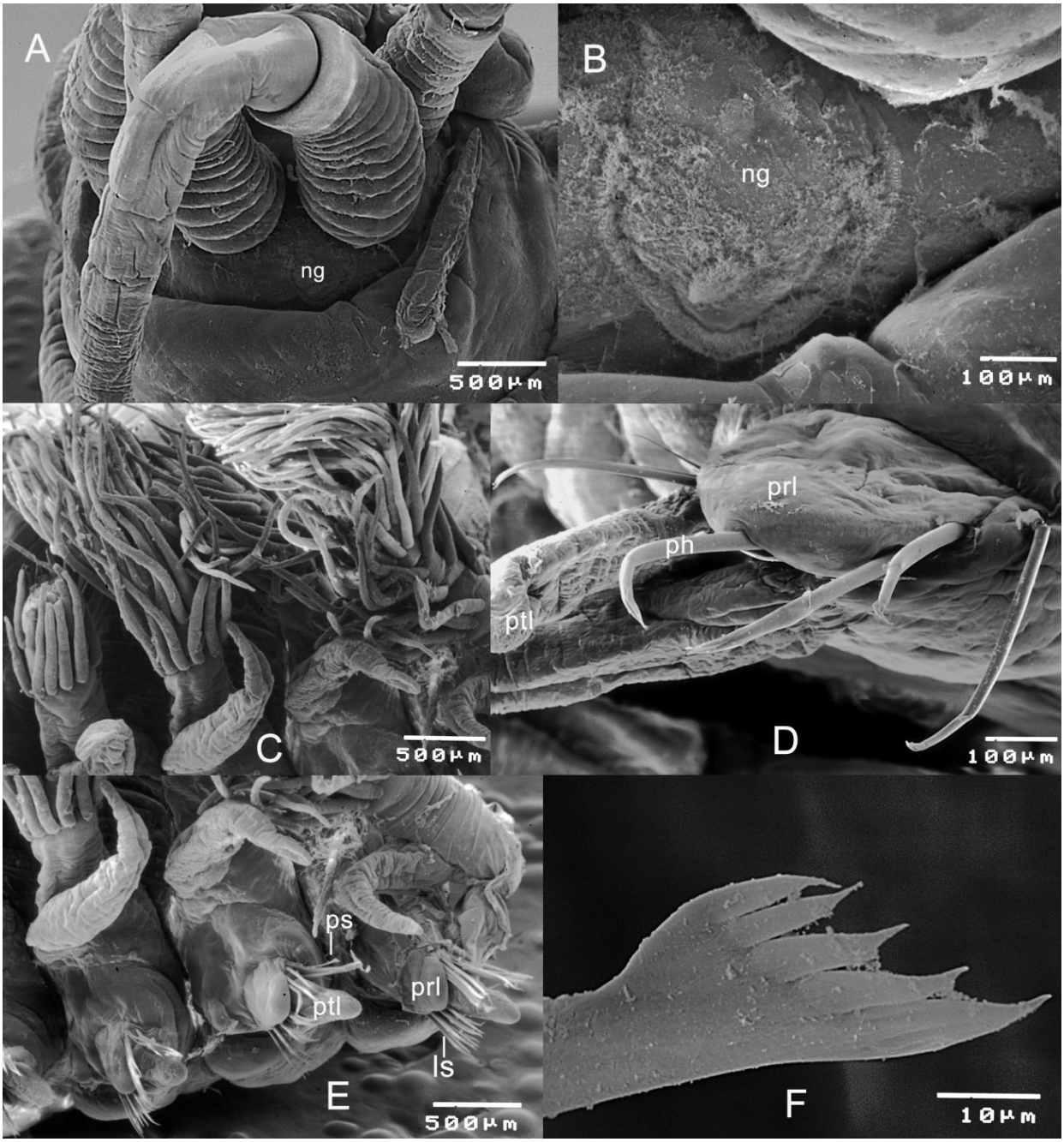

Figure 5 - Scanning electron micrographs of *Diopatra neapolitana*: A – prostomium, dorsal view; B – nuchal groove; C – branchiae; D – modified parapodium; E – parapodium of chaetigers 5, 6 and 7; F – pectinate chaeta. ls – limbate chaeta; ng – nuchal groove; ph – pseudocompound hook; prl – prechaetal lobe; ps – pectinate chaeta; ptl – postchaetal lobe.

Ventral lobes are absent in *D. marocensis*. In *D. neapolitana* they are present from chaetiger 5 to about 20–25. They are most distinct on setigers 10–20, then shifting more dorsally, forming the new presetal lip by chaetiger 20–25. Spiralled

branchiae appeared from chaetigers 4 or 5 in both species. They were best developed from chaetigers 5 to 9 with 6–9 whorls in *D. marocensis* and from chaetigers 7 to 9 with 14–18 whorls in *D. neapolitana* (Figs. 3C and E; 4E; 5C; cf. Table 1). The branchial filaments became gradually slender towards posterior chaetigers and were absent from chaetigers 30–41 in *D. marocensis* and from 56–70 in *D. neapolitana* (cf. Table 1).

Concerning the chaetae, *Diopatra marocensis* presented bidentate and *Diopatra neapolitana* uni- to bidentate pseudocompound hooks (Figs. 4F and 5D) with pointed hoods and two rows of blunt small spines along their shafts and limbate chaetae, on the first four modified parapodia. In the remaining parapodia limbate and pectinate chaetae appeared together with bidentate subacicular hooks from chaetigers 13–15 in *D. marocensis* and 19–25 in *D. neapolitana*. Limbate and pectinate chaetae have different morphology in the two species. Limbate chaetae in *D. neapolitana* were coarsely serrated along almost the whole borderline while in *D. marocensis* they were only serrated on the shelf. Pectinate chaetae have 11–20 teeth in *D. marocensis* with slender base up to the tip (Fig. 4H) while *D. neapolitana* presented 5–10 wider teeth, slenderer in the tip than in the base (Fig. 5F; cf. Table 1).

The pygidium was similar in both species with two pairs of anal cirri.

2.3.2 *Diopatra micrura*, sp nov.

Figs. 6–11; Tables 2–4

Type material. *Holotype*: MNHN MB29-000166, Sta. RA4 (Nov-09) (incomplete specimen, 51 mm long (61 chaetigers), 3.3 mm wide).

Paratypes: MNHN MB29-000167, Sta NS (1); MNHN MB29-000171, Sta.RA1 (1); MNHN MB29- 000168, Sta TS14B (1); MNHN MB29-000170, Sta TS23C (1); MNHN MB29-000169, Sta TS24 (1); MNCN 16.01/11627, Sta. TS1A (1); MNCN 16.01/11628, Sta.TS3A (1); MNCN 16.01/11629, Sta. TS4A (1); MNCN

16.01/11630, Sta. TS22 (1); MNCN 16.01/11631, Sta. TS25 (1); AM W36251, Sta. NS (1); AM W36252 Sta. TS13 (1); AM W36253 Sta. TS23 (2); AM

W36254, Sta. TS23C (1); AM W36255, Sta. RA2 (1); DBUA-01140.01, Sta. TS1B (1); DBUA-01141.01, Sta. TS12 (1); DBUA-01142.01, Sta. TS12A (1); DBUA-01143.01, Sta. TS14C (1.); DBUA-01144.01, Sta TS23A (1).

Etymology. The striped antennae of the new species evoke the pattern of the coral snakes *Micrurus* spp., hence the name *Diopatra micrura*, **sp. nov.**

Morphological description. Length of complete preserved specimens from 1.7 to 7.8 cm, number of chaetigers from 70 to 97; width of 10th chaetiger from 0.6 to 4.5 mm without parapodia. Some incomplete specimens regenerating anterior end of body (paratype AM W36252); one specimen posterior end.

Overall colour of living specimens greenish dorsally, cream ventrally. Antennostyles and palpostyles with very characteristic transverse brown bands, 4–8 on antennae and 2–4 on palps (Figs 6A–C, 7A). Frontal lips whitish with brown pigment at base and ceratophores with brown rings (Fig 6B). Prostomium with brown pigment; area of nuchal grooves paler (Figs 6B, C). Peristomium with brown pigment (Fig. 6C, 7A), peristomial cirri cream. Additionally, anterior 10–15 chaetigers with small iridescent white spots (Fig. 6C) and following chaetigers with iridescent transverse white line (Fig. 6A). Laterally, from chaetigers 1–4 to 13–23 two brown patches, one on each side (Figs 6C, 7A). Branchiae green, parapodia cream (Fig. 6D); dorsal cirri with iridescent white spots.

In preserved individuals, the body is cream with two brown patches laterally on each segment up to chaetigers 13–23 (Fig. 7A). Lack of coloration in middle of each chaetiger forming “white” line along body (Fig. 7A). Brown pigmentation of antennae, palps, ceratophores, prostomium, frontal lips and peristomium noticed in living specimens still present. Prostomium anteriorly rounded with subulate frontal lips (Figs 8A, B). Ceratophores of antennae and palps with proximal rings and longer distal ring, holotype with 14 proximal rings, other specimens with 12–15 rings (Figs 6A–C, 7A, 8A). Antennostyles relatively long, tapering to distal end, ending in fine point; in holotype laterals reaching chaetiger 9, median reaching chaetiger 5, in other specimens 6–13 and 4–10 respectively; palpostyles shorter, reaching chaetiger 2 in holotype, 2–4 in other specimens. Length of antennae quite variable, apparently unrelated to size of specimens (based on width of 10th

chaetiger) (Fig. 9A). Sensory buds present on antennostyles and palpostyles forming 12–14 irregular longitudinal rows (Fig. 8C). Sensory buds slightly raised, with pores forming circles (Fig. 8D). In addition, randomly distributed sensory buds on ceratophores, frontal lips, upper lips, prostomium, peristomial cirrus, peristomium and branchiae (Figs 8G, H). Nuchal grooves crescentic (Fig. 8E).

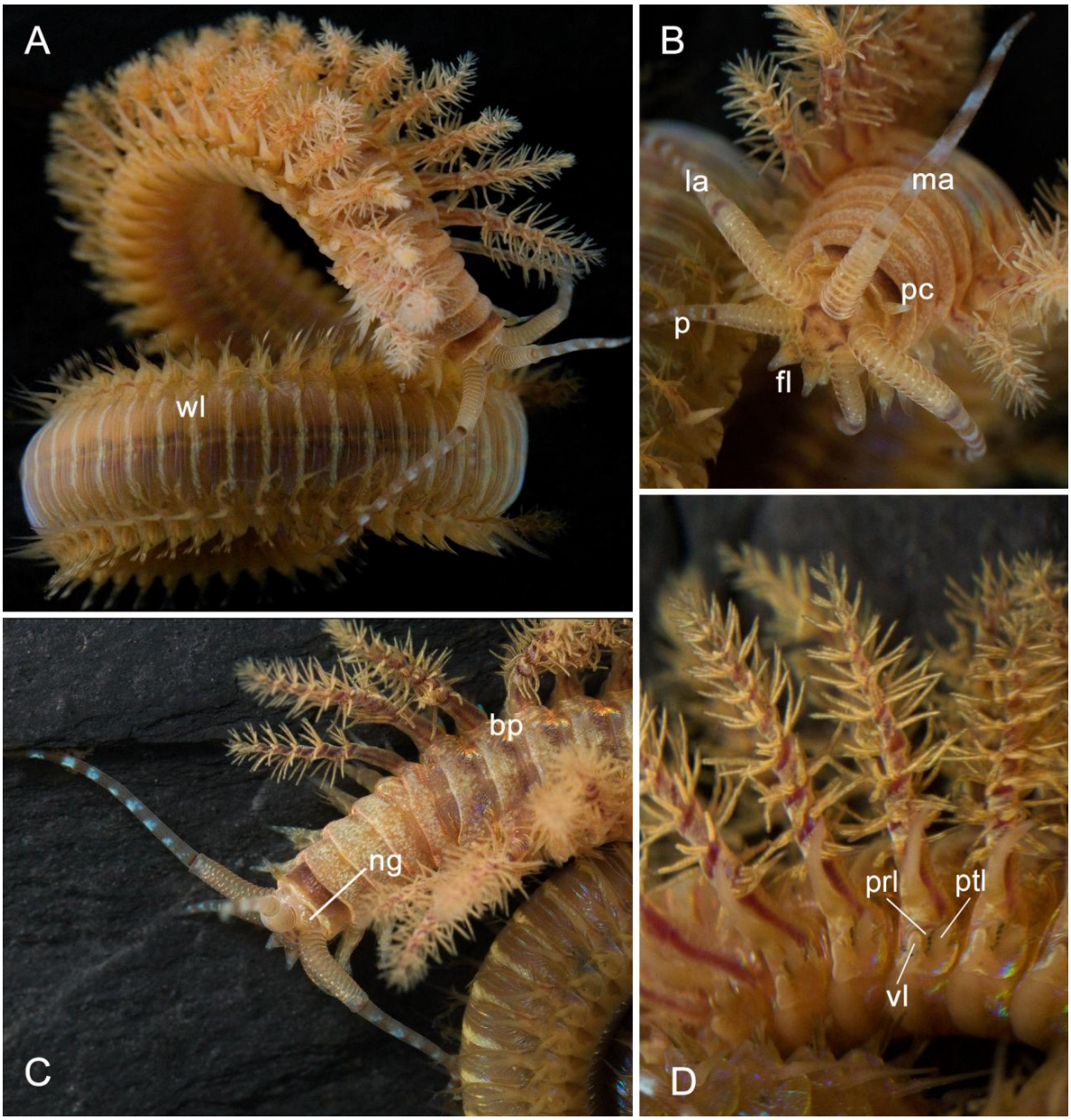

Figure 6 - Live specimen of *Diopatra micrura* **sp. nov.**: A, general view; B, prostomium, frontal view; C, anterior end, dorsal view; D, anterior unmodified parapodia and branchiae, lateral view; (bp) brown patch; (fl) frontal lip; (la) lateral antenna; (ma) median antenna; (ng) nuchal groove; (p) palp; (pc) peristomial cirrus; (prl), prechaetal lobe; (ptl) postchaetal lobe; (vl) ventral lobe; (wl) white transverse line.

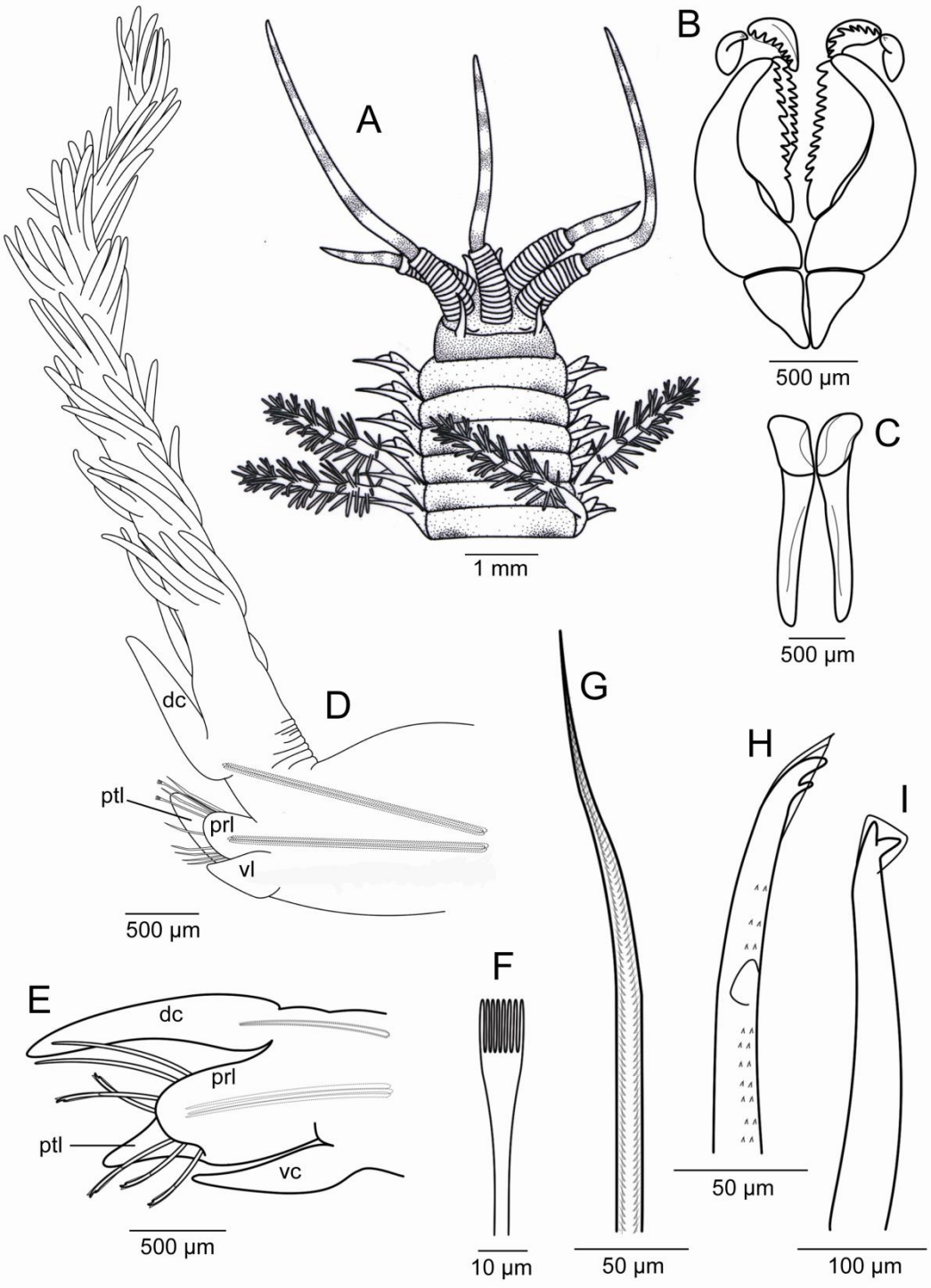

Figure 7 - *Diopatra micrura* **sp. nov.**: A, anterior end, dorsal view; B, maxillary apparatus, dorsal view; C, mandibles, ventral view; D, parapodium of chaetiger 6, anterior view; E, parapodium of chaetiger 1, anterior view; F, pectinate chaeta; G, limbate chaeta; H, pseudocompound hook; I, subacicular hook; (dc) dorsal cirrus; (prl) prechaetal lobe; (ptl) postchaetal lobe; (vl) ventral lobe; (vc) ventral cirrus.

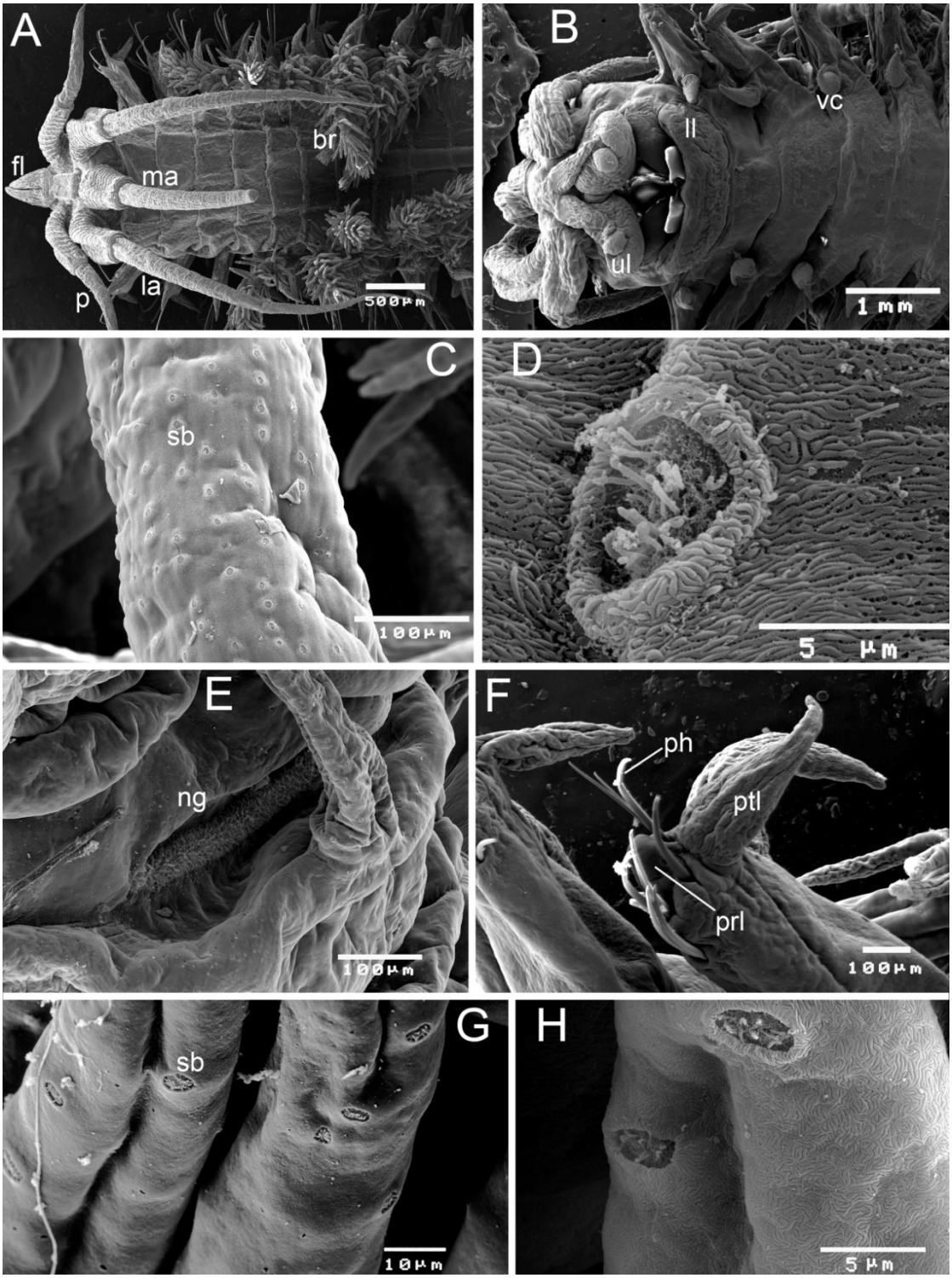

Figure 8 - Scanning electron micrographs of *Diopatra micrura* **sp. nov.**: A, anterior end, dorsal view; B, anterior end, ventral view; C, rows of sensory buds on antenna; D, enlarged sensory bud of antennae; E, peristomium and nuchal groove area; F, modified parapodium; G, sensory buds on branchiae; H, enlarged sensory bud of branchiae; (br) branchiae; (fl) frontal lips; (la) lateral antenna; (ll) lower lip; (ma) median antenna; (ng) nuchal groove; (p) palp; (ph) pseudocompound hook, (prl) prechaetal lobe; (ptl) postchaetal lobe; (sb) sensory bud; (ul) upper lip; (vc) ventral cirrus.

Peristomium as long as first chaetiger, bearing pair of peristomial cirri, about twice as long as peristomium (Fig. 7A). First four modified parapodia (chaetiger 1 to 4) projecting laterally and slightly anteriorly, slightly longer than following non-modified, laterally projecting parapodia (Figs 8B, F). Prechaetal lobes rounded, present up to chaetigers 7 – 11, postchaetal lobes subulate (Fig. 8F), becoming gradually smaller towards posterior region but still distinct till end of body. Ventral lobes present on chaetiger 5 to 14–20, subulate to ovate (Fig. 6D); most distinct on chaetigers 6–15, then shifting more dorsally, forming new prechaetal lip by chaetiger 20–25. Dorsal cirri subulate, becoming more slender posteriorly; ventral cirri cirriform on first 4 chaetigers. Spiralled branchiae from chaetiger 4 in holotype, chaetigers 4 or 5 in paratypes, best developed from chaetigers 6 to 9 with 8–14 whorls, reaching to prostomium when anteriorly extended (Figs 6A, C); decreasing gradually towards posterior end, absent from chaetiger 45 in holotype, chaetigers 32–55 in other specimens, depending on size of specimens (Fig. 9B). Branchial filaments fine and short, only slightly longer than width of branchial stem (Figs 6D, 7D).

Modified parapodia with 1–2 slender upper limbate chaetae and 5–6 bidentate pseudocompound hooks (Fig. 7E). Hooks with moderately long pointed hoods (Figs 7H, 10A) and two rows of small spines along their shafts (Figs 4H, 7B). Remaining parapodia with limbate and pectinate chaetae (Figs 7D, 10C, D). Pectinate chaetae flat, with 5–10 long teeth, ending in slender tips (Figs 7F, 10E); limbate chaetae with narrow serrated wings, overall spiny (Figs 7G, 10F).

Starting from chaetiger 11 in holotype, chaetigers 8–13 in other specimens, lower limbate chaetae replaced by 2 thick bidentate subacicular hooks with translucent guards (Fig. 7I). Slope of the regression line of start of subacicular hooks very close to nil, indicating a non-significant relationship to size of specimens (Fig. 9B).

Pygidium with two pairs of anal cirri; dorsal pair about as long as the last six chaetigers, ventral pair about as long as the two last chaetigers.

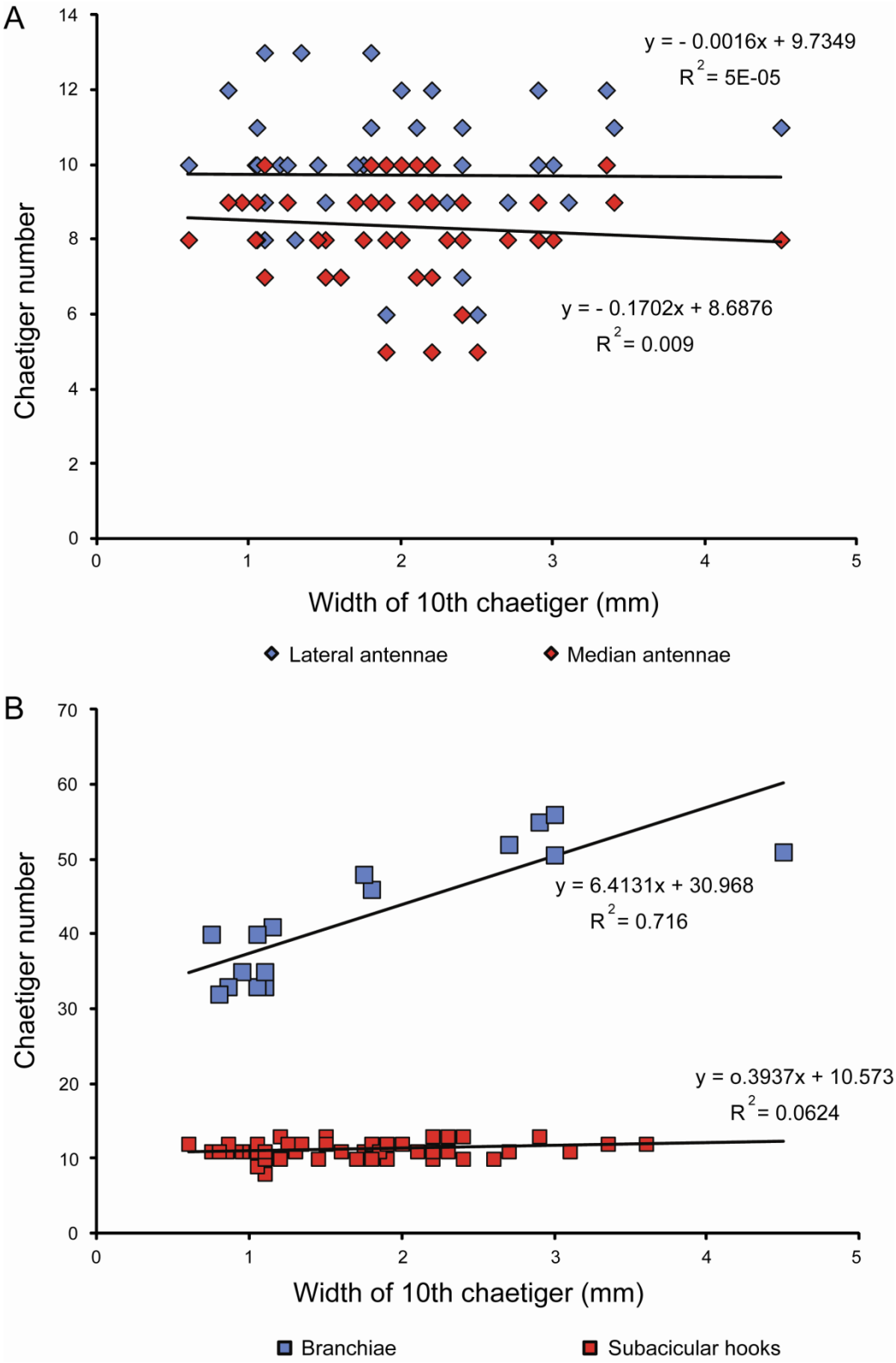

Figure 9 - *Diopatra micrura* sp. nov.: A, relationship between body width (chaetiger 10, without parapodia) and length of lateral and median antennae. B, relationship between body width (chaetiger 10, without parapodia) and chaetigers of last branchiae and first subacicular hooks.

Mandibles (Fig. 7C) weakly sclerotised, with slender shafts and strongly calcified cutting plates. Maxillae moderately sclerotised (Fig. 7B). Maxillary formula (based on 9 paratypes): Mx I= 1+1; MxII = 8–10 + 8–11; Mx III = 8–11 + 0; Mx IV = 5–8 + 7–11; Mx V = 1 + 1.

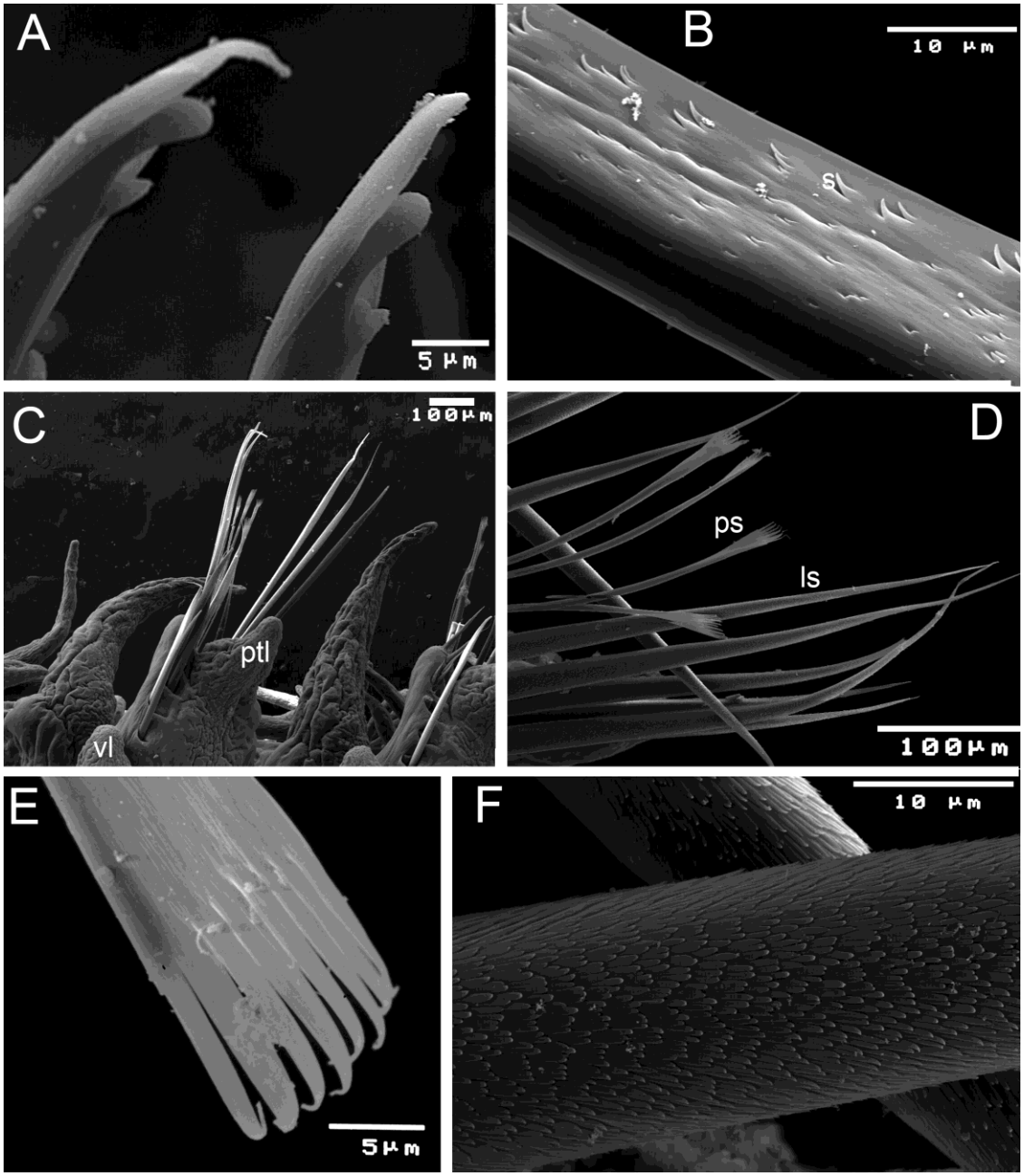

Figure 10 - Scanning electron micrographs of *Diopatra micrura* **sp. nov.**: A, distal ends of pseudocompound hooded hooks; B, spines of pseudocompound hooded hook; C, parapodium of chaetiger 9; D, pectinate and limbate chaetae of parapodium 13; E, pectinate chaeta; F, spines of limbate chaetae; (s) spines; (ls) limbate chaeta; (ps) pectinate chaeta; (ptl) postchaetal lobe; (vl) ventral lobe.

Tube characteristic of genus, cylindrical with soft inner secreted layer and outer layer of debris, fragments of sea grass, algae and shells.

Remarks. The intraspecific variability of the major morphological characters of *Diopatra micrura* is summarised in Table 2 and the comparison with other European *Diopatra* species in Table 3.

Table 2. Intraspecific variability of the most important morphological characters of *Diopatra micrura* **sp. nov.** (SD = Standard deviation, N = number of individuals observed).

Character	Range	Mean	SD	N
Length, complete preserved specimens) (cm)	1.7-7.8	5.4	3.25	3
Number of chaetigers, complete specimens	70-97	86.0	14.18	3
Width of 10th chaetiger without parapodia (mm)	0.6-4.5	1.91	0.79	77
Lateral antennophores (number of rings)	12-15	14.42	0.75	85
Median antennophore (number of rings)	12-15	13,46	0,91	83
Palpophores (number of rings)	12-15	13.36	0.93	87
Lateral antennae (reaching chaetiger)	6-13	9.64	1.71	66
Median antenna (reaching chaetiger)	4-10	8.25	1.43	67
Palps (reaching chaetiger)	2-4	2.26	0.48	72
Peristomial cirrus/peristomium (length ratio)	1.5-2.8	1.84	0.30	63
First branchiae (chaetiger)	4-5	4.53	0.5	89
Last branchiae (chaetiger)	32-55	42.94	8.11	18
Branchial whorls (maximum number)	8-14	10.92	1.79	75
First subacicular hooks (chaetiger)	8-13	11.24	1.08	57
Last prechaetal lobes (chaetiger)	7-11	9.36	1.16	42
Last ventral lobes (chaetiger)	14-20	16.83	1.80	37
Pectinate chaetae (number of teeth)	5-10	7.00	0.98	45

Table 3 - Comparison of morphological descriptors (mean ± standard deviation) measured in *Diopatra micrura* sp. nov., *D. neapolitana* and *D. marocensis* from Portuguese waters and in the Arcachon specimens (presumably *Diopatra* sp. A mentioned in Berke et al., 2010). Range between brackets.

Character	*D. neapolitana* (present study)	*D. marocensis* (present study)	*D. micrura* sp. nov.	Specimens from France	
				Diopatra sp.	*D. neapolitana*
Colour (living specimens)	greenish	pinkish	greenish	-	-
Length, complete preserved specimens (cm)	36.39 ± 13.50	8.93 ± 1.98	5.4 ± 3.25	-	-
Number of chaetigers, complete specimens	269.20 ± 31.16	141.69 ± 22.8	86.00 ± 14.18	-	-
Average width of 10th chaetiger (mm)	7.08 ± 1.68	2.97 ± 0.66	1.90 ± 0.78	7.08 ± 0.66	5.25 ± 1.06
Frontal lips (shape)	subulate	subulate to ovate	subulate	subulate	subulate
Nuchal grooves (shape)	rounded	crescentic	crescentic	rounded	rounded
Sensory buds on antennae and palps (numbers of rows)	20-22	16-18	12-14	-	-
Ceratophores (number of rings)	15-16	6-9	12-15	9-11	14-16
First branchiae (chaetiger)	4.46 ± 0.51 (4-5)	4.14 ± 0.36 (4-5)	4.54 ± 0.5 (4-5)	4.33 ± 0.52 (4-5)	4.5 ± 0.71 (4-5)
Last branchiae (chaetiger)	64.40 ± 3.53 (56-70)	33.77 ± 2.96 (26-38)	42.82 ± 8.34 (32-55)	52.67 ± 4.73 (49-58)	51.0 ± 4.24 (48-54)
Branchial whorls (maximum number)	14-18	6-9	8-14	9-12	15
First subacicular hooks (chaetiger)	19-25	13-15	8-13	15-17	19
Last prechaetal lobes (chaetiger)	15-20	6-10	7-11	16-19	18-20
Ventral lobes	present	absent	present	absent	present
Pectinate chaetae (number of teeth)	5-10	11-20	5-10	25-32	6-9

(To be continued.)

Table 3 – (continued.)

Character	*D. neapolitana* (present study)	*D. marocensis* (present study)	*D. micrura* sp. nov.	Specimens from France	
				Diopatra sp.	*D. neapolitana*
Specimens (number examined)	35	35	88*	6	2
Locality	Aveiro	Aveiro, Guia	Aveiro, Guia	Arcachon Bay, Marennes Olérons	Arcachon Bay
Habitat	Intertidal	Intertidal/Subtidal	Subtidal/Intertidal	Intertidal	Intertidal

The multivariate analysis of the morphological data is shown in Figure 11. The groups of individuals belonging to the various species form distinct clusters (Fig. 11, upper graph), represented by well isolated clouds of points in the ordination diagram (Fig. 11, lower graph). The PCA axis 1 and 2 comprehend 91.3% of the total variance. *Diopatra neapolitana* opposes *D. marocensis* on the ordination axis 1, with *D. micrura* occupying a transition position, on the positive pole of axis 2. Most of the *Diopatra* specimens from France form a distinct cluster, isolated in the negative pole of axis 2 but closer to *D. marocensis*. This cluster includes five specimens from Arcachon Bay and a single specimen from Marennes Oléron (Fig. 11). Nevertheless, two specimens from the Arcachon Bay are plotted together with the cluster of *Diopatra neapolitana*, indicating the existence of at the least two species in this Bay. The morphological descriptors most strongly correlated with PCA axis 1 were the number of branchiae whorls (r = - 0.92), the number of rings in the ceratophores (r = - 0.90) and the presence-absence of ventral lobe in the parapodia 5–20 (r = - 0.85). The chaetiger where the subacicular hooks start (r = - 0.72), the width of the 10th chaetiger (r = - 0.70) and the number of teeth in the pectinate chaetae (r = - 0.53), were the variables strongly correlated with PCA axis 2, the latter especially related to the *Diopatra* sp. individuals from Arcachon Bay (Fig. 11).

2.3.3. Genetic analysis

A 702-bp COI fragment and a 525-bp 16S fragment were successfully obtained from 14 individuals of *D. micrura*, 45 individuals of *D. marocensis* and of *D. neapolitana* and 2 of *Diopatra* sp. individuals from Arcachon. COI and 16S nucleotide sequences from *D. micrura* **sp. nov.**, *D. marocensis* and *D. neapolitana* were deposited at EMBL database, under the accession numbers: *D. marocensis* 16S – J473306, COI – FJ646632 and GQ456165, *D. neapolitana* 16S – EU878538, COI – EU878539 and GQ456164 and 16S – GQ456163 and COI – GQ456161 and GQ456162.

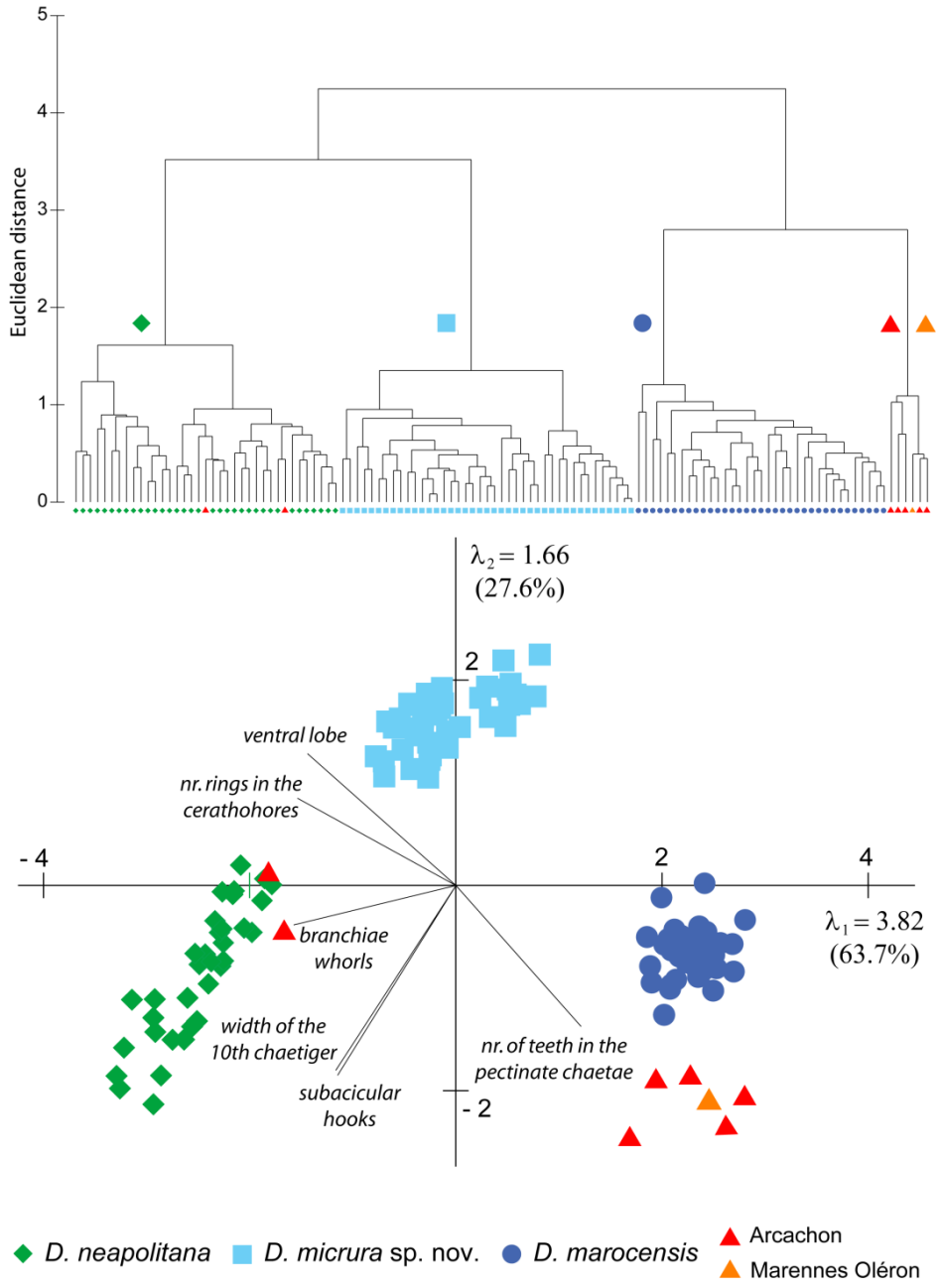

Figure 11 - Classification (upper graph) and ordination (lower graph) analysis of specimens of European *Diopatra* species, according to morphological descriptors. Most of the specimens obtained in France (Arcachon Bay and Marennes Oléron) form an isolated cluster, corresponding to a fourth species, but also include individuals belonging to *D. neapolitana*.

For the 16S gene, all individuals of *D. micrura* displayed identical nucleotide sequence but in the case of the COI gene, one individual from Ria de Aveiro presented a base alteration, at position 276, where a nucleotide adenine was

replaced by a thymine (ATA to TTA), corresponding to an amino acid alteration (methionine to leucine). All specimens sampled on the shelf off the Tagus Estuary shared the same nucleotide sequence.

In *D. marocensis*, all individuals analysed displayed identical nucleotide sequences for the 16S gene. For the COI gene two haplotypes were found. Two individuals from Guia presented a base alteration at position 404, where a nucleotide adenine was replaced by a cytosine (TCA to TCC), corresponding to a silent alteration with no amino acid change. All specimens of *D. marocensis* from Ria de Aveiro had the same nucleotide sequence.

For *Diopatra neapolitana*, two haplotypes were also observed in COI gene. A base alteration occurred at position 560, where a nucleotide cytosine was replaced by a thymine (CTC to CTT), corresponding to a silent substitution with no amino acid change. In this case, 24 individuals had a CTC codon and 21 the CTT. For the 16S gene, all individuals of *D. neapolitana* shared the same nucleotide sequence.

The percentage of nucleotides divergence of the 16S and COI genes between *D. micrura* and *D. marocensis* was 15% and 17%, respectively (nucleotide substitution). For *D. micrura* and *D. neapolitana*, the divergence was 16% for COI and 12% for 16S. Between *D. marocensis* and *D. neapolitana*, the divergence was 14% for 16S and 17% for COI.

For COI, deduced amino acid sequence comparison between the three species revealed that *D. micrura* differs from *D. marocensis* in six amino acids and from *D. neapolitana* in two amino acids, for one haplotype, and in three for the other, revealing 2.59% and 1.08% of divergence, respectively. *D. neapolitana* and *D. marocensis* differ in 4 amino acids, showing 1.74% of divergence. The majority of the differences in nucleotides between these species occurred at the third position of the codon and therefore corresponds to silent alterations.

Comparing COI and 16S genes of *Diopatra* sp. from Arcachon Bay with *D. neapolitana*, *D. marocensis* and *D. micrura*, the percentage of nucleotides divergence varied between 17% and 19% in the case of the COI and between 16% and 19%, for 16S gene (nucleotide substitution).

The nucleotide sequence of 16S and COI genes of *D. neapolitana* and *D. marocensis* were compared with the nucleotide sequence of *Diopatra aciculata* deposited in the EMBL database (Struck et al., 2006; COI: AY838867, 16S: AY838826). The mean divergence values (nucleotide substitution), between *D. marocensis* and *D. aciculata*, were 18% and 14% for COI and 16S, respectively. These values were similar to the ones obtained in this study when comparing *D. marocensis* and *D. neapolitana*. However, the percentage of divergence between *D. neapolitana* and *D. aciculata* was of 5% and 1% for COI and 16S, respectively.

The phylogenetic analysis from both genes (Fig. 12) separates the *Diopatra* species into four clades, however, *D. neapolitana* and *D. aciculata*, are very close, in a sister clade.

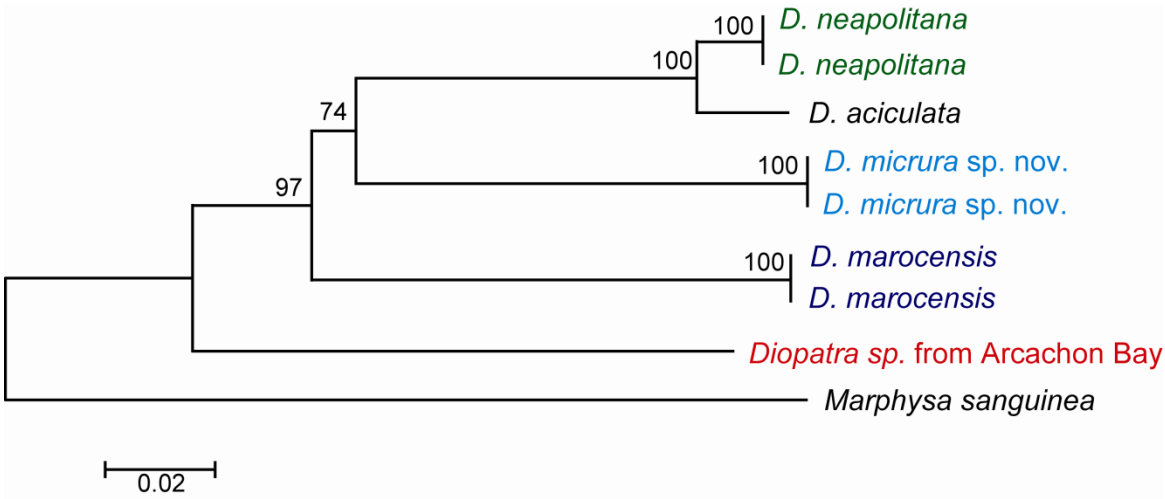

Figure 12 - Phylogenetic analysis of the data set containing the 16S and COI sequences of *Diopatra* species. Numbers near the nodes indicate the percent bootstrap values. The branch length indicator represents 0.02 substitutions per site.

2.3.4. Distribution and habitat of *Diopatra micrura* and *Diopatra marocensis*

2.3.4.1. *D. micrura*

Diopatra micrura occurs along the western and southern Portuguese coast. Specimens were collected in Ria de Aveiro, near the mouth, intertidally and on the

adjacent shelf area (A), on the shelf off Nazaré (B), in Guia, off the Tagus Estuary (C), and near the Guadiana river mouth (D) (Fig. 2, Table 4).

The species seems to have a preference for fine sand and shallow waters (Table 4). In the shelf area off the Tagus estuary, it was found in 22 of the 30 sites comprising the annual monitoring program of this area, carried out since March 1994. *Diopatra micrura* has been found in every annual sampling campaign, in sites ranging from 40 to 60 metres depth, on fine and very fine sand, with fines content up to 25% of total sediment. In the Aveiro shelf, *D. micrura* was found in 8 of 22 sites sampled in 2002, always close to 15 metres depth and in fine and very fine sand with less than 5% fines content. In the Nazaré shelf, the species was found at 37 metres depth, on fine sand with 7% fines. On the southern coast, it was found in fine and very fine sand with less than 5% fines, ranging from 4 to 10 metres depth. Finally, in Ria de Aveiro the species was found in the intertidal region, together with *D. marocensis* and *D. neapolitana*, in very fine sand with close to 25% fines content.

Table 4. List of sites where *Diopatra micrura* **sp. nov.** was sampled. AS—Shelf off Aveiro; NS—Nazaré; RA—Ria de Aveiro; GS—Shelf off Guadiana River; TS—Shelf off Tagus Estuary; SVT - Total Volatile Solids (organic matter).

Station number	Latitude (ºN)	Longitude (ºW)	Depth (m)	Date	Sediment type	Fines content (%)	SVT (%)
TS1	38° 39' 45.840"	9° 25' 40.440"	40	Mar-94	Silty fine sand	9.03	2.71
TS1A	38° 39' 45.840"	9° 25' 40.440"	40	Apr-01	Silty fine sand	9.03	2.71
TS1B	38° 39' 45.840"	9° 25' 40.440"	40	Oct-03	Silty fine sand	9.03	2.71
TS2	38° 40' 29.340"	9° 27' 59.580"	40	Jan-97	Clean fine sand	3.4	1.43
TS3	38° 40' 33.600"	9° 28' 11.640"	40	Jan-97	Clean fine sand	4.47	1.41
TS3A	38° 40' 33.600"	9° 28' 11.640"	40	Oct-01	Clean fine sand	4.47	1.41
TS3B	38° 40' 33.600"	9° 28' 11.640"	40	Oct-03	Clean fine sand	4.47	1.41
TS4	38° 40' 20.820"	9° 28' 7.440"	45	Jan-97	Clean fine sand	3.52	1.12
TS4A	38° 40' 20.820"	9° 28' 7.440"	45	Oct-01	Clean fine sand	3.52	1.12
TS4B	38° 40' 20.820"	9° 28' 7.440"	45	Oct-03	Clean fine sand	3.52	1.12
TS5	38° 40' 25.080"	9° 27' 50.640"	40	Jan-97	Clean fine sand	3.36	1.39
TS5A	38° 40' 25.080"	9° 27' 50.640"	40	Apr-97	Clean fine sand	3.36	1.39
TS6	38° 40' 37.620"	9° 28' 22.080"	40	Jan-97	Clean fine sand	5.32	1.49
TS6A	38° 40' 37.620"	9° 28' 22.080"	40	Oct-98	Clean fine sand	5.32	1.49
TS6B	38° 40' 37.620"	9° 28' 22.080"	40	Apr-01	Clean fine sand	5.32	1.49

(To be continued.)

Table 4 – (continued.)

Station number	Latitude (ºN)	Longitude (ºW)	Depth (m)	Date	Sediment type	Fines content (%)	SVT (%)
TS7A	38° 40' 51.060"	9° 29' 0.180"	40	Oct-97	Silty medium sand	10.06	1.41
TS8	38° 41' 20.220"	9° 29' 51.600"	40	Jan-97	Clean coarse sand	0.81	1.28
TS8A	38° 41' 20.220"	9° 29' 51.600"	40	Oct-02	Clean coarse sand	0.81	1.28
TS9	38° 40' 1.560"	9° 29' 41.640"	34	Jan-97	Clean fine sand	1.57	1.4
TS10	38° 40' 37.620"	9° 27' 53.940"	38	Apr-97	Clean fine sand	2.07	1.30
TS10A	38° 40' 37.620"	9° 27' 53.940"	38	Oct-02	Clean fine sand	2.07	1.30
TS11	38° 40' 20.100"	9° 28' 27.120"	45	Apr-97	Silty fine sand	5.08	1.45
TS12	38° 40' 16.920"	9° 27' 38.460"	40	Apr-97	Clean fine sand	3.71	1.34
TS12A	38° 40' 16.920"	9° 27' 38.460"	40	Oct-01	Clean fine sand	3.71	1.34
TS13	38° 40' 38.820"	9° 27' 27.540"	26	Apr-97	Clean fine sand	3.88	1.61
TS14	38° 40' 2.640"	9° 27' 0.480"	40	Apr-97	Silty fine sand	5.20	1.50
TS14A	38° 40' 2.640"	9° 27' 0.480"	40	Oct-99	Silty fine sand	5.20	1.50
TS14B	38° 40' 2.640"	9° 27' 0.480"	40	Oct-03	Silty fine sand	5.20	1.50
TS14C	38° 40' 2.640"	9° 27' 0.480"	40	Jan-06	Silty fine sand	5.20	1.50
TS15	38° 40' 56.100"	9° 28' 8.400"	26	Oct-99	Clean fine sand	3.87	1.28
TS15A	38° 40' 56.100"	9° 28' 8.400"	26	Oct-02	Clean fine sand	3.87	1.28
TS16	38° 40' 51.060"	9° 29' 0.180"	40	Apr-01	Silty medium sand	10.06	1.41
TS17	38° 39' 53.640"	9° 28' 29.940"	60	Oct-01	Silty very fine sand	14.29	2.21
TS18	38° 39' 51.060"	9° 27' 16.500"	50	Oct-02	Silty very fine sand	24.87	3.39
TS19	38° 40' 15.109"	9° 26' 30.275"	35	Oct-02	Clean fine sand	2.83	1.2
TS20	38° 40' 4.881"	9° 24' 9.258"	27	Oct-02	Clean fine sand	4.50	1.6
TS21	38° 39' 49.183"	9° 23' 43.095"	26	Oct-02	Silty very fine sand	11.12	1.6
TS22	38° 40' 6.180"	9° 27' 23.880"	40	Oct-03	Clean fine sand	4.10	1.84
TS23	38° 39' 49.200"	9° 23' 43.080"	26	Oct-03	Silty fine sand	11.05	2.52
TS23A	38° 39' 49.200"	9° 23' 43.080"	26	Oct-04	Silty fine sand	11.05	2.52
TS23B	38° 39' 49.200"	9° 23' 43.080"	26	Oct-07	Silty fine sand	11.05	2.52
TS23C	38° 39' 49.200"	9° 23' 43.080"	26	Oct-08	Silty fine sand	11.05	2.52
TS23D	38° 39' 49.200"	9° 23' 43.080"	26	Sep-09	Silty fine sand	11.05	2.52
TS24	38° 39' 54.600"	9° 26' 14.340"	40	Oct-04	Silty fine sand	5.33	1.73
TS24A	38° 39' 54.600"	9° 26' 14.340"	40	Jan-06	Silty fine sand	5.33	1.73
TS25	38° 40' 7.440"	9° 27' 48.420"	45	Jan-06	Clean fine sand	4.56	1.54
TS26	38° 40' 33.720"	9° 28' 45.900"	45	Oct-07	Silty fine sand	5.82	1.60
AS1	40° 40' 50.637"	8° 46' 45.085"	15	Dec-02	Clean very fine sand	1.6	0.7
AS2	40° 41' 11.718"	8° 46' 32.926"	15	Dec-02	Clean very fine sand	1.61	0.84
AS3	40° 41' 4.855"	8° 47' 8.918"	18	Dec-02	Clean very fine sand	1.88	0.97
AS4	40° 40' 38.579"	8° 46' 26.623"	13	Dec-02	Clean very fine sand	1.67	0.92
AS5	40° 41' 0.196"	8° 46' 15.844"	13	Dec-02	Clean very fine sand	1.34	0.72
AS6	40° 41' 56.762"	8° 46' 6.409"	15	Dec-02	Clean very fine sand	1.18	0.71
GS1	37° 10' 21.119"	7° 28' 2.219"	12	May-07	Silty fine sand	5.03	-

(To be continued.)

Table 4 – (continued.)

Station number	Latitude (ºN)	Longitude (ºW)	Depth (m)	Date	Sediment type	Fines content (%)	SVT (%)
GS2	37° 8' 52.440"	7° 24' 59.281"	10	May-07	Very silty very fine sand	46.14	-
NS	39° 50' 43.901"	9° 6' 40.799"	37.2	Apr-08	Silty fine sand	6.65	0.94
RA1	40° 38' 28.896"	8° 44' 0.276"	Intertidal	Oct-08	Silty fine sand	24.7	4.3
RA2	40° 38' 28.896"	8° 44' 0.276"	Intertidal	Mar-09	Silty fine sand	24.7	4.3
RA3	40° 38' 28.896"	8° 44' 0.276"	Intertidal	Sep-09	Silty fine sand	24.7	4.3
RA4	40° 38' 28.896"	8° 44' 0.276"	Intertidal	Nov-09	Silty fine sand	24.7	4.3

2.3.4.2. *Diopatra marocensis*

At present *Diopatra marocensis* has been identified along the western and southern Portuguese coast (Fig. 2): in 23 of the 30 sites sampled in the Guia area, in the vicinity of a submarine outfall (April 1997–October 2007), in 5 of the 107 sites sampled in the Lagoon of Óbidos (July 2002), in 4 sites of Ria de Aveiro and in 1 of the 92 sites of a survey covering Olhão to Vila Real de St° António. The sediment types, percentage of fines and percentage of total volatile solids (TVS) of the sites where *D. marocensis* was found are presented in Table 5. The species has been found in a range of sediments (mud, very fine sand, fine sand and medium sand), with fines ranging from 71.3% to 0.8% and total volatile solids from 6.5% to 1.1%.

2.3.5 *Diopatra* species distribution in Ria de Aveiro

Fig. 13 shows the distribution of the three *Diopatra* species along Ria de Aveiro. *D. neapolitana* shows the widest distribution. *D. marocensis* was found cohabiting with *D. neapolitana* in two sites (and one also with *D. micrura*) located near the entrance of Ria de Aveiro, in 2008, and in four sites in the 2009 survey which seems to indicate that it is dispersing throughout the system. *D. micrura* was only found in two sites, near the entrance and coexisting with the other two species in one site, and cohabiting with *D. neapolitana* on the other locality (Fig. 13). The study of the reproductive biology of *D. marocensis,* next chapter, was

based in samples from near the entrance of Ria de Aveiro, where the population is well established (Fig. 13).

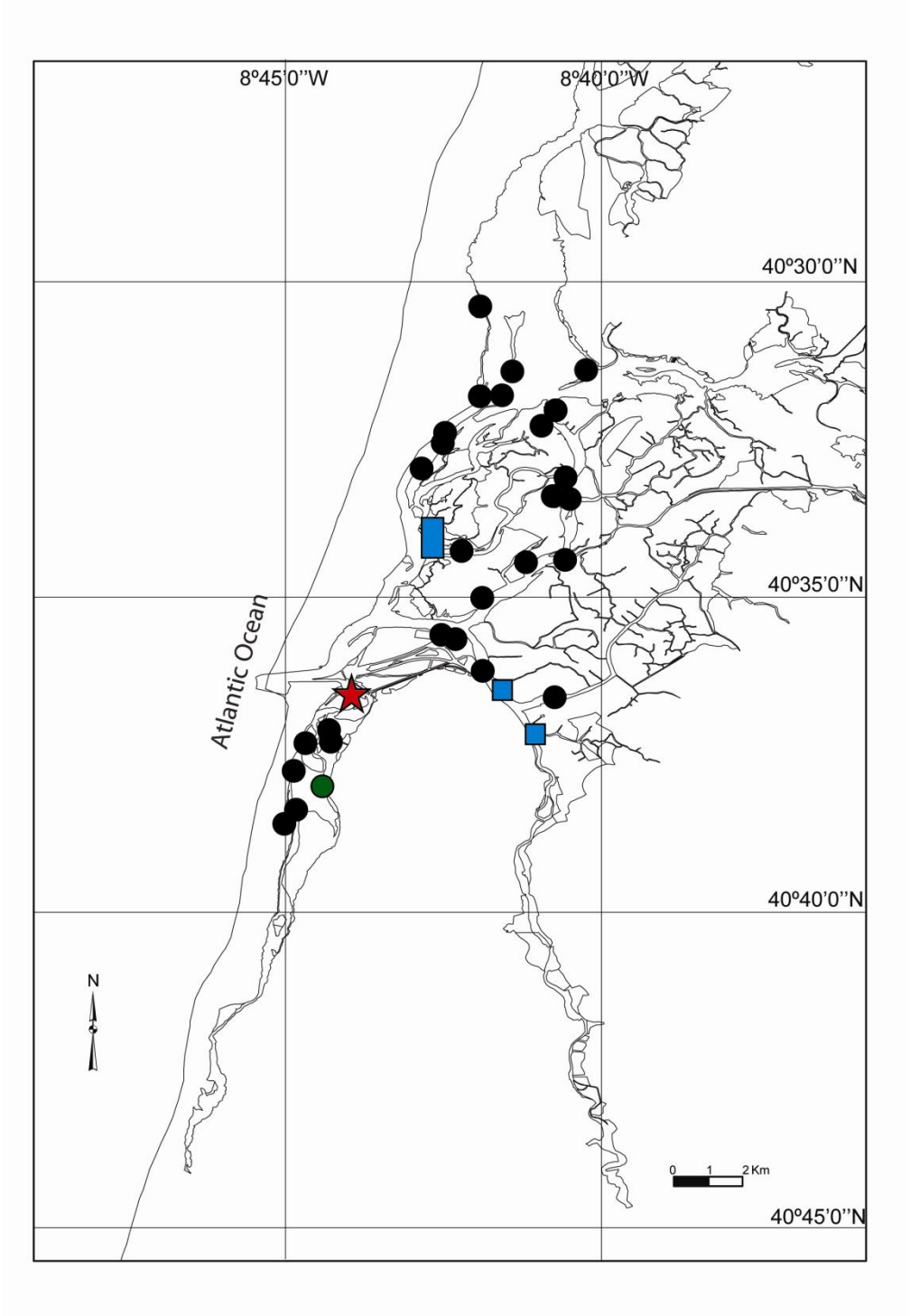

Figure 13 - Areas in Ria de Aveiro where *Diopatra* species were found. (● - *D. neapolitana*; ● - *D. neapolitana* and *D. micrura*; ■ - *D. neapolitana* and *D. marocensis*; ★ - *D. neapolitana*, *D. marocensis* and *D. micrura*. Surveys in October 2008 and August 2009.

Table 5 - Sediment characteristics from the sampling areas where *Diopatra marocensis* was found (TVS – Total Volatile Solids).

Locality	Sediment type	Fines content (%)	TVS (%)
Ria de Aveiro	Very silty fine sand	34.4	4.5
	Silty fine sand	24.7	4.3
Lagoon of Óbidos	Mud	71.3	5.0
	Very silty very fine sand	48.5	6.5
	Silty fine sand	15.8	1.9
	Clean medium sand	0.8–2.1	0.6
Guia	Clean fine sand	3.5–4.91	1.1–1.46
	Silty fine sand	5.1–12.4	1.2–1.6
	Silty very fine sand	16.5	2.2
Olhão	Clean fine sand	1.0	–

2.4. Discussion

The present work presents a morphological and genetic comparison between the species *Diopatra neapolitana, Diopatra marocensis*, and *Diopatra micrura* sp. nov., recording the presence of *D. marocensis* on European coasts, and describing *D. micrura* as new species. *D. marocensis* was first mentioned outside the Moroccan coast in the annual meeting of the Portuguese Ecological Society (Pires et al., 2008) and latter, in the lagoon of Óbidos by Rodrigues et al. (2009) and Berke et al. (2010), being its distribution along the Southern Portuguese coast referred for the first time by Rodrigues et al. (2009).

The available temporal data allow tracing back to 1997 the presence of *Diopatra marocensis* on the western coast of Portugal, where the species was initially misidentified as *Diopatra neapolitana*. In fact, after reanalysis of the collected material, no specimens of *D. neapolitana* were registered in this shelf

area off the Tagus estuary. In Ria de Aveiro, the present study is the first concerning *Diopatra* species and there is no material from older surveys to analyze. As such, it is not possible to ascertain if the presence of *D. marocensis* in this system is only recent. *D. marocensis* was observed in two localities in October 2008 and in four a year later. It presents a well-established population close to the entrance, and seems to be dispersing throughout the system. This is coherent with the temporal spreading pattern of this species close to a marine outfall, as reported by Rodrigues et al. (2009), suggesting that the species could be augmenting its geographical distributional range, and once installed, spreading the local settlement area. This should be important to follow up and realize if it will out compete with *Diopatra neapolitana* that has a much higher economical value, and at the present is the most abundant and wide spread *Diopatra* species in Ria de Aveiro.

D. micrura was a species found essentially in subtidal sites. In Ria de Aveiro, *D. micrura* was only found in the lower intertidal of two localities. It is possible that these three species could be found in more subtidal areas.

Arias et al. (2010) reported that *D. marocensis* had been present in the estuary of Villaviciosa since May 1976, demonstrating that it is not a recent species in the estuary. The same authors stated also that between 1976 and 2000, the species had a wider distribution in the central and outer basins of the estuary, and that in 2010 the species was only found in a specific area of the central basin. During the last years an increase of the population of *D. neapolitana* was observed (Arias et al., 2010). The same authors explained that the changes in the density of *Diopatra* spp. in Villaviciosa estuary are probably direct and indirect consequences of anthropogenic disturbances experienced by the estuary mainly in its outer basin in recent years.

The wide distribution of *D. marocensis* along the Portuguese coast, the proximity of the Moroccan coast and also the way *D. neapolitana* species is commercially exploited in Portugal is not in favour of an accidental introduction by bait trade, as suggested by Berke et al. (2010). Bait trade of *Diopatra* in Portugal is mainly for a local market or otherwise only for exportation. The exploited populations are all natural and at the present there are no cultivated areas based upon imported specimens.

Although the population of *D. marocensis* in the shelf area off the Tagus estuary is installed mainly over clean fine sand, it was found in sediments ranging from mud to clean medium sand, a wider variety than that known for *Diopatra neapolitana* which is common in muds and muddy sands (Fauvel, 1923, Bellan, 1964 and Gambi et al., 1998).

The new species, *Diopatra micrura,* was found on the western and the southern coast of Portugal, in fine or very fine sand with less than 30% of fines content, from the intertidal region up to 50 metres depth. *Diopatra micrura* coexists with other *Diopatra* species, namely *D. neapolitana* and *D. marocensis* but it is much less common and was never recorded in densities as high as those of the other two species. This study also showed the coexistence between *D. neapolitana* and *Diopatra* sp. in the Bay of Arcachon, from intertidal specimens collected in 2009, contrary to the opinion expressed by Berke et al. (2010) who set the Northern limit distribution of *D. neapolitana* on the Spanish French border.

The presence of *D. micrura* off the Tagus Estuary, on the western coast of Portugal, can be traced back as far as 1994, where the species has been regularly recorded in monitoring samples taken yearly. In that same coastal area, *D. marocensis* has shown an increase in density and distributional area over the last five years (Rodrigues et al., 2009), but this has not, so far, excluded *D. micrura*. This study shows that *D. micrura* can be distinguished from its European congeners by morphological and genetic characteristics and proposes a key to the European species of *Diopatra*.

Diopatra micrura is most closely related to *D. neapolitana*, a species with which it occurs sympatrically in Ria de Aveiro. Both species possess ventral lobes on parapodia 5–20. These lobes have only been observed in *D. monroviensis* Augener, 1918 from West Africa and in *D. aciculata* Knox & Cameron, 1971 from Australia. The latter is morphologically very similar to *D. neapolitana* but shows some genetic isolation as was discussed by Rodrigues et al. (2009). *Diopatra micrura* can easily be distinguished from the other species by its striped antennostyles and palpostyles, crescentic rather than rounded nuchal grooves, much smaller adult size and more anterior start of the subacicular hooks.

Furthermore, there are differences in the construction of the tubes. The characteristic tubes of *D. monroviensis* have a thick outer layer of sand with even thicker ridges every centimetre or so, while those of the other three species lack the ridges and have also some fragments of seagrass, algae and shells attached.

D. neapolitana, *D. marocensis* and *D. micrura* sp. nov. are clearly three different species showing morphological distinctions such as size, nuchal grooves, number of the rings in the ceratophore, chaetae (Fauvel, 1923, Paxton et al., 1995 and Dagli et al., 2005) and colour pattern, corroborated by genetic evidence concerning the mitochondrial DNA genes, 16S and COI genes. All individuals analysed of each species displayed identical nucleotide sequence for the 16S gene while for the COI gene two haplotypes were found.

For the COI gene, deduced amino acid sequences of both species differ in four amino acids, which correspond to a 1.74% divergence. However, they are replaced by others of the same chemical group (Stryer, 1999) and therefore the sequences are translated in proteins of the same family that will have the same function.

In the molecular studies of the 16S and COI genes, all individuals of *D. micrura* displayed an identical nucleotide sequence for the 16S gene but, in the case of the COI gene, one individual from Ria de Aveiro presented a base alteration at position 276. This corresponded to a replacement of adenine by thymine, and an amino acid alteration occurred. However as these amino acids belong to the same chemical group (Stryer, 1999) the sequences are translated in proteins of the same family that will have the same function.

The mitochondrial genes, COI and 16S rRNA, are considered conserved genes, but the relative nucleotide divergence that we obtained between the four *Diopatra* species – averaging 17.5% and 15.4% respectively – is usual among different species of polychaetes. In fact, in the case of 16S rRNA, in the dorvilleid genus *Ophryotrocha* the mean sequence divergence is 12% (Dahlgren et al., 2001); in the syllid genus *Autolytus* it is 21%, with a range of 28–17%, based on 16 species (Nygren & Sundberg, 2003), within the *Palola* genus the mean divergence is 12.4% (Schulze, 2006); for the genus Eunice it is 14% (based on 3

species sequences deposit in the GenBank; range: 13–17%) and for the genus *Lumbrinereis* is 13% (based on 4 species sequences; range: 12–14%). In the case of COI, sequence divergence in the terebellid genus was 20% for two *Loimia* species and 19% for two *Amphitrite* species (Schulze, 2006); for the *Palola* genus, the mean divergence is 14.5% (Schulze, 2006); in the genus *Dorvillea* the nucleotide mean sequence divergence is 22% (based on 3 species sequences; range: 20–23%) and in the genus *Lumbrinereis* is 20% (based on 4 species sequences; range: 18–22%).

These comparisons suggest that the genetic variation between *Diopatra neapolitana* and *Diopatra marocensis* and *D. micrura* is within a normal range for polychaetes. But the genetic comparison between *D. neapolitana* and *D. aciculata* is below, 5% and 1% for COI and 16S rDNA, respectively, emphasizing the similarity between these two species.

The phylogenetic relationship analysis of the European *Diopatra* species revealed four clades, representing four distinct species of *Diopatra*, emphasising the validity of *D*. *micrura* as a distinct species. However, *Diopatra aciculata* and *D. neapolitana* are in a sister clade. Dagli et al. (2005) and Paxton (1993) stated that *D. aciculata* is very similar to *D. neapolitana* concerning its morphological appearance and chaetae types. The results presented in this work emphasize the similarities between these two species at a genetic level. Despite the fact that some differences occur they can be expected between distant populations of a species (note that the EMBL database information for *D. aciculata* concerns an Australian population). However being the COI and 16S conservative genes in the mtDNA genome, better in showing differences than similarities, further studies using for instance, faster mtDNA markers or quicker nuclear markers, like microsatellites, are desired, to lead to a reliable conclusion about the taxonomic validity of *D. aciculata*.

The multivariate analysis of morphological descriptors between the *Diopatra* species analysed showed a very good separation between the groups of individuals from different species and allows similar conclusions regarding the

validity of the four European species of *Diopatra*, for which the following key is proposed:

1 Antennae with transverse brown bands; parapodia 5–20 with ventral lobes; 12–16 rings on ceratophores 2

- Antennae without transverse brown bands; ventral lobes absent; 6–11 rings on ceratophores 3

2 Antennae with 4–8 transverse brown bands (Fig. 3A–C), small species, up to 10 cm long, 4.5 mm wide; subacicular hooks starting from chaetiger 8–13; crescentic nuchal grooves ..... *D. micrura,* **sp. nov.**

- Antennae with single median brown band; large species, up to 40 cm long, 9 mm wide; subacicular hooks starting from chaetiger 19–25, rounded nuchal grooves .....*D. neapolitana*

3 Dorsum with mid-dorsal brown patch, forming line along anterior part of body; nuchal grooves crescentic; parapodia with single postchaetal lobes; pectinate chaetae with 11–20 teeth, crescentic nuchal grooves ... *D. marocensis*

- Dorsum without pigment; nuchal grooves rounded; parapodia 1–5 with double postchaetal lobes; pectinate chaetae with 25–32 teeth, rounded nuchal grooves .....*Diopatra* sp. from Arcachon Bay

Chapter 3

Reproductive biology of *D. neapolitana* and *D. marocensis* in Ria de Aveiro

3.1 Introduction

Conti et al. (2005) reported that *D. neapolitana* releases the eggs and sperm into the water column and Bhaud and Cazaux (1987) that it develops planktonic lecithotrophic larvae. Although the spawning of this species has never been observed in nature, Bhaud and Cazaux (1987) and Conti and Massa (1998) described several developmental phases based on data obtained by artificial fertilization and culture of the larvae. These authors showed that the larvae were lecithotrophic and free-swimming.

Fadlaoui et al. (1995) observed larvae of *D. marocensis* within maternal tubes and considered that they develop directly in the parental tube. These authors classified the larval development of this species into three stages, based in the number of chaetigers and ciliation. Stage 1 includes atrochal larvae, stage 2 encloses larvae with 1 to 3 chaetigers still having ciliation and stage 3 includes larvae without ciliation. The species reaches the adult stage at about 4 mm of 10^{th} chaetiger width, has maximum egg diameter of 600 µm and brood size ranging from 20 to 100 eggs

The present study focuses on the gametes' characteristics, the reproductive period, larval development and the sex ratio of populations of *D. neapolitana* and of *D. marocensis* in Ria de Aveiro along two years of study. Understanding the life history aspects of *D. neapolitana* are important steps for management and conservation efforts which are aiming at a sustainable exploitation of the species.

3.2 Materials and Methods

3.2.1 Study area and sampling

This study was conducted in Ria de Aveiro, Northwestern Portugal (Fig. 14). Ria de Aveiro is a shallow estuarine water system, receiving water from several rivers (Fig. 14), with the Vouga River accounting for more than 50% of the freshwater input, resulting in a complex system of bays, channels and extensive intertidal sand and mud flats (Dias et al. 1999).

D. neapolitana and *D. marocensis* specimens were collected intertidally, monthly, with a shovel, at up to 30 cm deep. At least 50 *D. neapolitana* individuals were collected randomly from May 2007 April 2009. A minimum of 60 specimens of *D. marocensis* were collected from July 2008 and June 2010.

Individuals were collected during low tide and the *Diopatra* tubes were removed from the sediment.

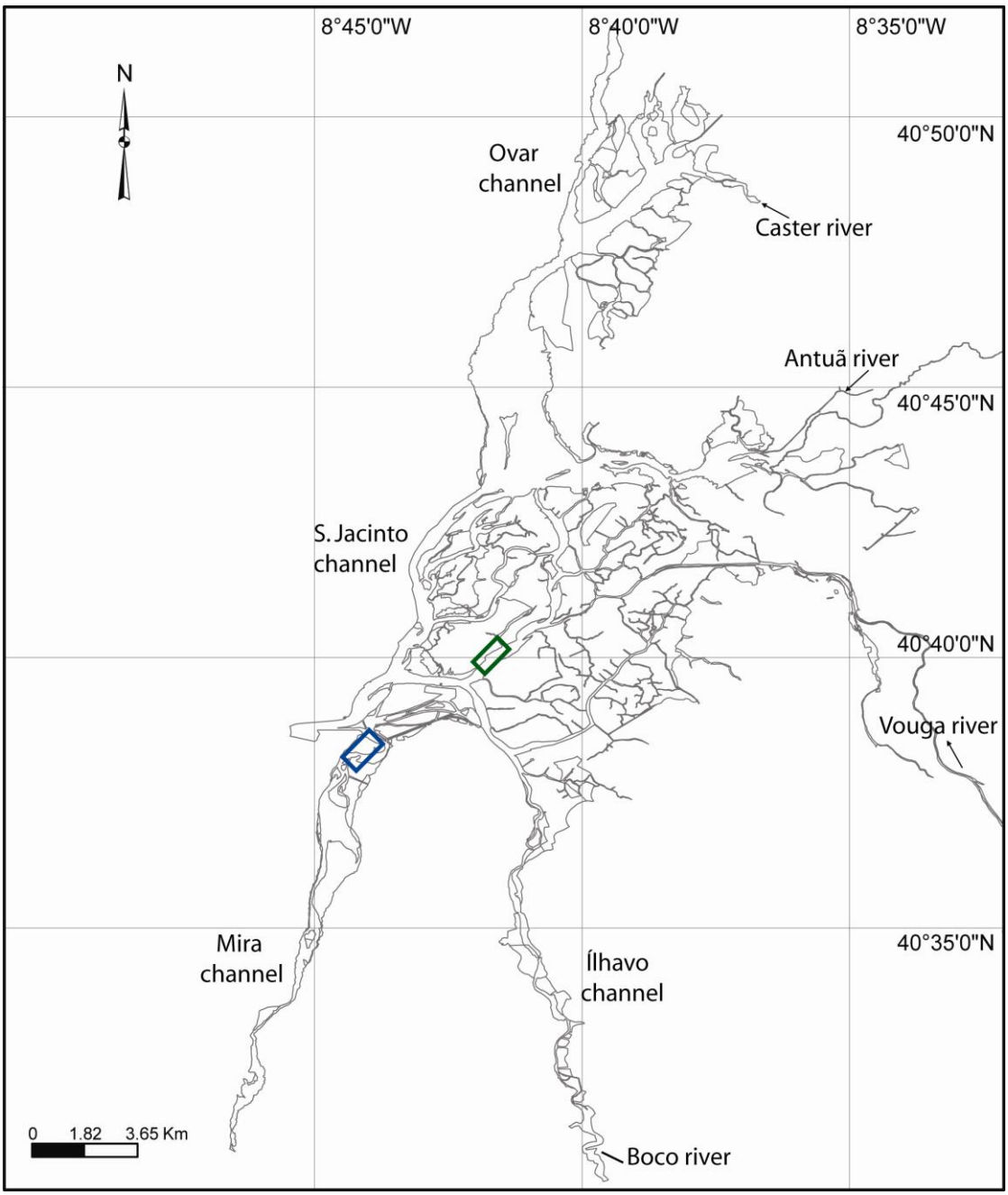

Figure 14 – General view of Ria de Aveiro showing sampling area (blue – sampling area of *D. marocensis*; green – sampling area of *D. neapolitana*).

3.2.2 Laboratory procedures

In the laboratory, *Diopatra* worms were individually removed from their tubes and washed in sea water.

3.2.2.1 *Dioptra neapolitana*

Each specimen was partly dissected to search for the presence of gametes and then fixed in 70% ethanol. Fixed specimens were measured for width at the 10^{th} chaetiger (without parapodia). Total length was measured in entire specimens (n=46, about 4% from the total of individuals). These morphological variables were only measured in individuals that were not seen to be regenerating.

The oocytes were extracted from females by dissection of the body cavity. The diameter of each oocyte was measured under a stereo-microscope (resolution 50x) using an ocular micrometer (precision of 0.01mm). For each female, the diameter of 100 oocytes was measured. During the periods with a larger number of mature individuals (April to August) oocytes were measured at least in 12 females. In the remaining study period, oocytes were measured in all the females collected, as their number was below 12. In some cases only 2 to 4 females with oocytes in the coelom cavity were sampled. In total, oocytes from 332 females were measured. To count the total number of oocytes per female only complete specimens were used 12 in total. These were collected between May and December. During the study period fresh sperm in sea water was observed under a microscope (resolution 1000x).

3.2.2.2. *Diopatra marocensis*

Prior to conservation in 96% ethanol, each worm was carefully observed, to search for the presence of gametes (yellow oocytes are visible through the body wall in the coelomic cavity and chaetigers with sperm are cream, almost white).

Fifteen females per month were examined for oocytes size, with 50 oocytes measured per female. The total number of oocytes was counted in complete

females. The oocyte diameter was measured under a stereomicroscope (resolution 50x) using an ocular micrometer (precision of 0.01mm).

Monthly, from December 2008 to June 2010, about 10 tubes with adults were fixed in 4% formalin and dissected under a stereomicroscope, to observe the presence/absence of eggs or larvae. When present, they were counted and measured (egg diameter and larvae total length and number of chaetigers). The parental females were measured and dissected to check for the presence of the gametes in the coelom cavity.

Morphological data were obtained in specimens that were not regenerating: width of the 10th chaetiger (without parapodia), the number of chaetigers with branchiae and the first and last chaetiger with gametes. In whole specimens (n=628, 42.4% from the total of individuals), the number of chaetigers was registered and the total length measured.

3.2.3 Fertilization *in vitro* of *D. neapolitana*

Specimens were collected from the study area and kept in the laboratory for at least two months. They were maintained at 22 ºC and at salinity in the range 30-35. Salinity was measured with a handheld refractometer and expressed using the Practical Salinity Scale. To study larval development, artificial fertilization was performed, following the method described by Conti and Massa (1998) for *D. neapolitana*. Females and males were cut laterally and left in separated dishes with seawater for 10-15 min. to release the eggs and sperm. A portion of sperm was collected and added to the oocytes. The fertilized eggs were cultured at 22 ºC and 30-35 salinity. Seawater was changed daily.

The larval development observed in this study was analyzed following the descriptions of Bhaud and Cazaux (1987) and Conti and Massa (1998).

Once settled, the larvae were fed with homogenized cockles. At 4 days after fertilization, in the metatrocophore phase, the larvae were moved to an aquarium with fine sediment. The study of larval stages was carried out under an optical microscope.

3.2.4 Data analysis

The relationship between total length (L) and the width of the 10th chaetiger (W) was studied using second order polynomial simple regression analysis for *D. neapolitana*. This relationship was established from 46 complete individuals collected during the whole study period, according to the function $L = a+b_1W + b_2W^2$, forcing the model through the origin (a=0). SPSS software (Version 17) was used to test the overall significance of the model (F - test) and of the second order regression coefficient (b_2, t - test). The total expected body length of broken specimens was then determined from the measured width of the 10th chaetiger, using the respective regression function. This relationship was used to determine the expected shortest length of mature individuals.

The mean oocyte diameter (MOD) was calculated per female and per month for both species. For all months, Spearman correlation was calculated between MOD – size of the females and between MOD – number of chaetigers with oocytes, for *D. marocensis* and between MOD – total length for *D. neapolitana*.

The variance of oocyte diameter of *D. neapolitana* specimens in the period of gametogenesis inactivity (November to January) was statistically compared (F-test) to the period of gametes production (March to October).

For complete *D. marocensis* specimens, the Spearman correlation was calculated between the specimen size – first chaetiger with gametes, specimen size - number of chaetigers with oocytes and number of chaetigers with branchiae – first chaetiger with gametes.

The larvae of *D. marocensis* specimens found in the parental tubes were classified into development stages following Fadlaoui et al. (1995).

3.3 Results

3.3.1 Relationship between total length and width oh the 10th chaetiger for *Diopatra neapolitana*

A total of 1163 specimens were observed during the study period. From these only 46 were entire specimens, with total length ranging from 24 to 725 mm,

with the width of the 10th chaetiger varying between 1.9 and 10.88 mm, respectively (Fig. 15).

All observed specimens, entire and incomplete, had a 10th chaetiger width between 1.9 mm to 13 mm. The regression function relating the body length of the specimens (L, in mm) to the width of the 10th chaetiger (W, in mm) was statistically significant (F=1081.5; p<0.0001) and given by the expression $L=17.955W+4.209W^2$. The regression coefficient associated with W^2 (b_2=4.209) was also found to be significantly different from zero, validating the second order polynomial (t=6.945; p<0.0001). Under this regression model, the width of the 10th chaetiger explained 98% of the total length variance (R^2 adj = 0.98). This regression function was used to estimate the total length of broken specimens. The smallest female observed to be carrying oocytes had W=4.2 mm, corresponding to an estimated body length of 149.7 mm and the smallest male with sperm in the coelom had W=4.0 mm, corresponding to an expected body length of 139.2 mm.

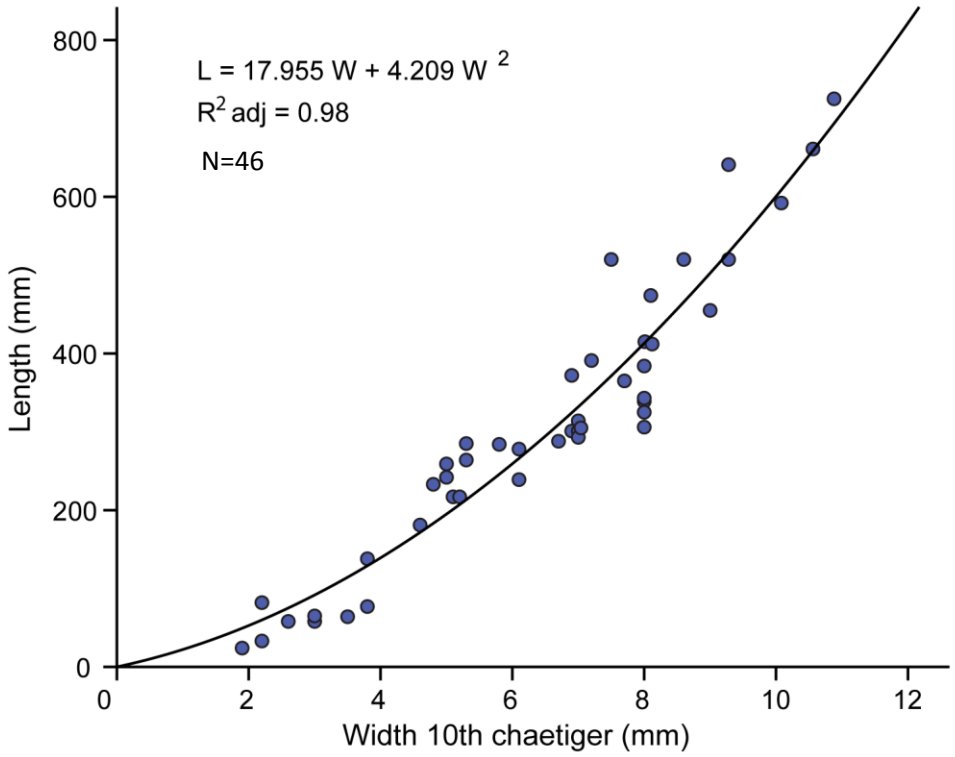

Figure 15 – *D. neapolitana*: Relationship between total length (L) and width of the 10th chaetiger (W)

3.3.2 Reproduction

3.3.2.1 Reproduction of *D. neapolitana*

The presence/absence of gametes was analyzed in a total of 1163 specimens, of which 320 were males, 332 females and 511 undetermined (with no gametes in the coelom). No external morphological differences were noticed between males and females. During the main reproductive period however males gained a cream color and females became greenish, mainly due to the gametes that are in the coelom.

The overall male:female sex-ratio was close to 1:1 from April to September. For the other months only very few individuals with gametes were captured and the sex-ratio was not determined.

The reproductive cycle of *D. neapolitana* can be inferred from the proportional variation of worms with gametes in the coelom, from the development of the oocytes size and from the number of oocytes in complete females (Figs. 16 and 17; Table 6). Some individuals with gametes inside the coelom were always found, but the percentages of males, females and of individuals without gametes varied (Fig. 16) and showed a consistent pattern in the two consecutive years. In February 2008 and February 2009 a single specimen with oocytes and a single specimen with sperm were found, respectively, whereas April to August presented a larger proportion of individuals with gametes (varying from 39.22 to 54.29 in females and 35.14 to 50.0 in males) (Fig. 16).

The smallest oocyte found in a female's coelom had a diameter of 40 µm and the largest had a diameter of 240 µm, with the mean for all specimens being 164.4 ± 40.8 µm. A number of small oocytes (<140 µm) was present in almost every month. It showed a peak in March and April, decreasing until September. Small oocytes were absent in some autumn/winter months (October, November and December) (Fig. 17). The decrease of small oocytes was parallel to the increase of the density of larger oocytes (Fig. 17). The mean oocyte diameter was not significantly correlated with the size of the females, measured through the width of the 10^{th} chaetiger (rho= 0.01; $p>0.05$). Mean oocyte diameter increased rapidly from March to May, and continued increasing slowly until January (Fig. 17). The

variance in oocyte size was significantly larger from March to October (s^2=1354) than during the winter months, from November to January (s^2=264; F= 5.1; p<0.001). This can be appreciated in figs. 17 and 18.

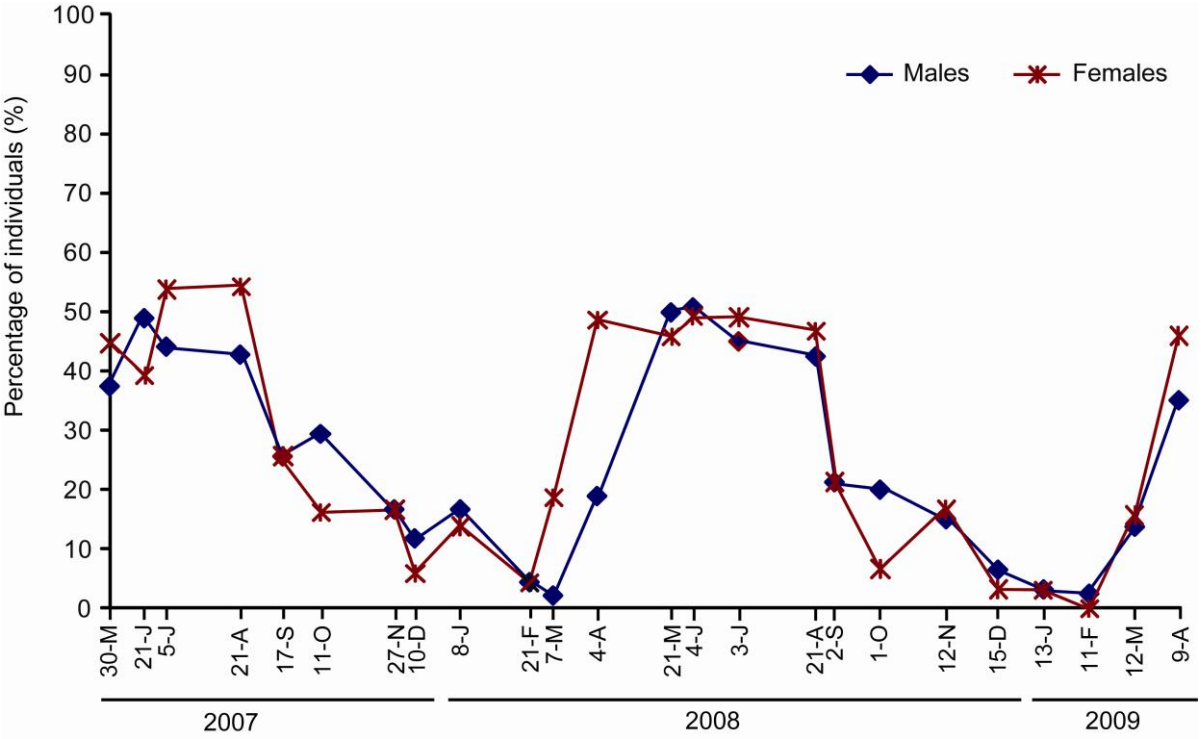

Figure 16 – Temporal development of males and females given as a percentage of the total number of specimens analyzed monthly. Only individuals with gametes were considered.

Females from November to January had mainly large oocytes, between 140 to 240 µm (Fig. 18). Nurse cells were observed in oocytes with a diameter up to 160 µm (Fig. 19A). They are attached to the immature oocytes with two strings measuring up to 230 µm long (mean = 177.5 ± 35.4 µm) containing up to 39 cells (mean 29.4 ± 4.5 µm) with 12 µm diameter. Oocytes larger than 160 µm did not have nurse cells attached (Fig. 19B).

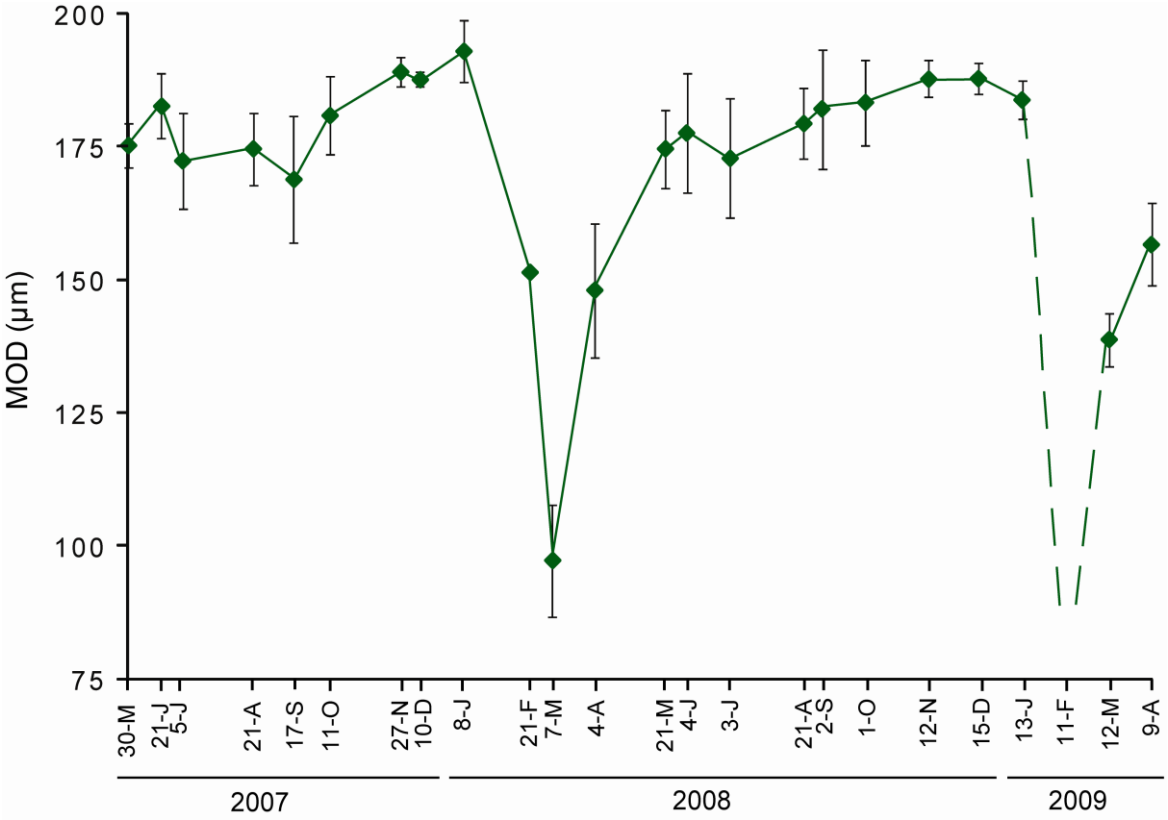

Figure 17 – Evolution of the mean oocyte diameter (MOD, µm), during the study period. No specimens with oocytes in the coelom were obtained in February 2009. The bars represent the standard deviation.

Table 6 – Mean number of oocytes in complete females in several months, with maximum and minimum numbers observed. The number of females analysed in each period is shown in brackets.

Months	Mean	Maximum	Minimum
May (2)	1,821,846.5	1,921,337	1,722,356
June (3)	453,885.7	553,874	378,496
August (3)	73131.5	78,260	68,003
September (2)	30,880.5	39,165	22,596
October (1)	20,190	-	-
December (1)	20,818	-	-

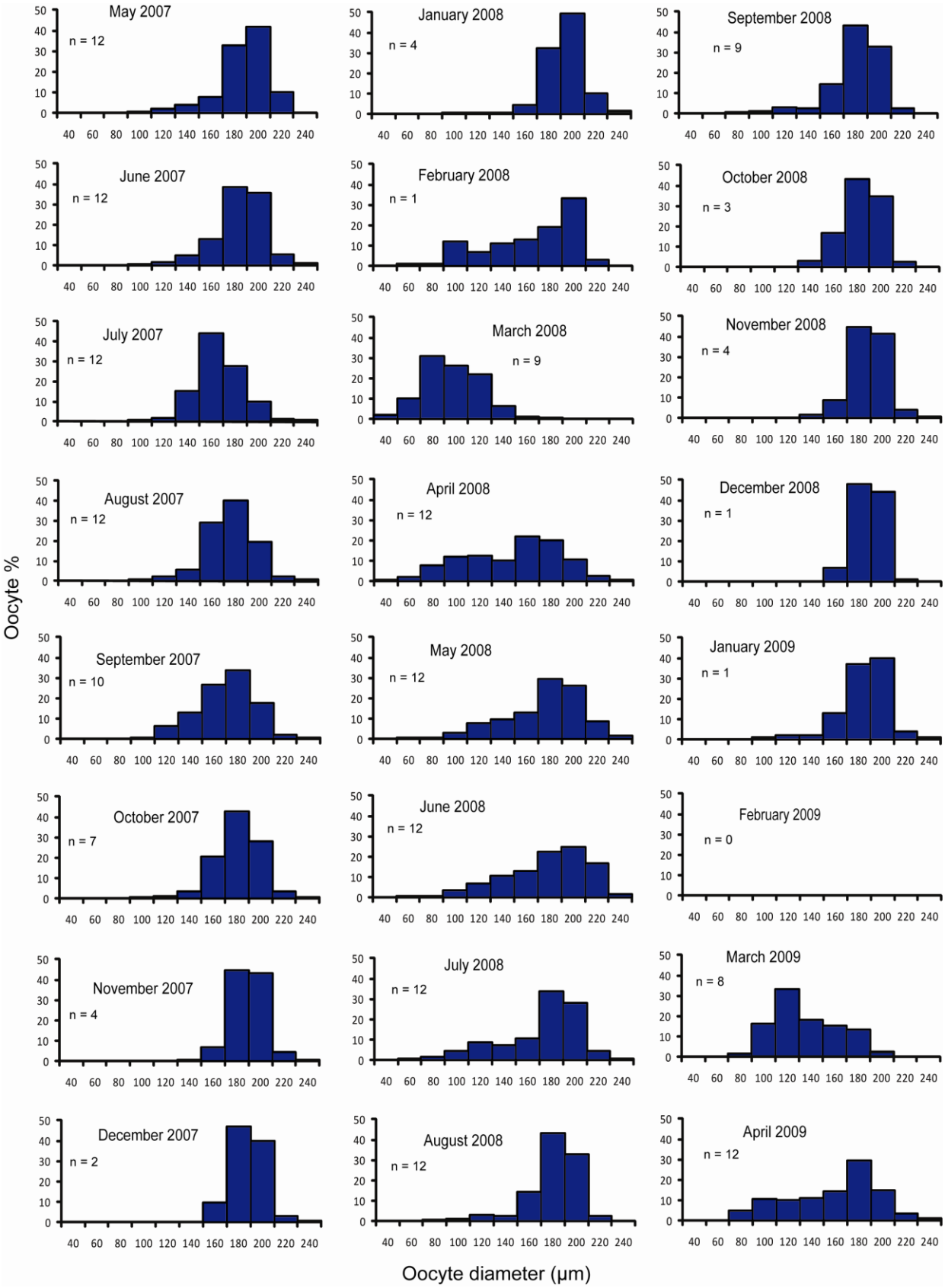

Figure 18 – Size-frequency distribution of oocytes of *D. neapolitana* during the study period (n = number of females observed).

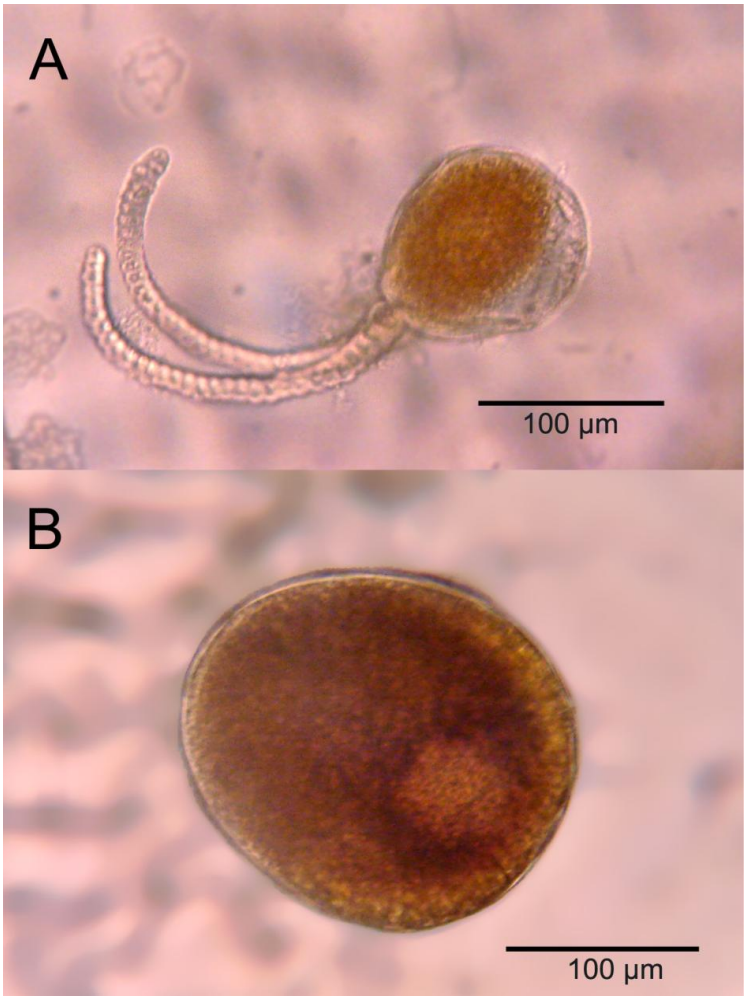

Figure 19 – Oocytes of *D. neapolitana*. A – Immature oocyte with nurse cells attached; B – Mature oocyte.

Sperm had a spherical, short, and rounded head with a long tail and were grouped in capsules in the coelom. When sperm were observed under the microscope, between May and August, the majority of the males contained spermatozoa with a mobile flagellum, moving actively in sea water. From October to January, spermatozoa had tails, but reduced mobility. Sperm were immobile during the others months.

The first chaetiger with gametes was variable. In females where the oocytes were observed they were between the 35th and the 70th chaetiger. In males, sperm were found from chaetiger 50 to 70. The mean chaetiger where gametes first appeared was 52.7 ± 8.6 for oocytes and 59.3 ± 7.3 for the sperm. No significant

correlation was found between the first chaetiger bearing gametes and the size of the individuals (rho=0.01; p>0.05). The gametes were distributed in the coelom till the end of the body.

From the months where it was possible to count the total number of oocytes per female (complete females), May and June, had the females with the highest number of oocytes in the coelom (Table 6).

3.3.2.2 Reproduction of *D. marocensis*

A total of 1482 specimens were observed during the study period. From these 628 were entire specimens, with total length ranging from 24 to 139 mm, with width of 10th chaetiger from 1.2 to 5.7 mm respectively, and total number of chaetigers from 75 to 214. The 10th chaetiger width ranged from 2.5 to 5.7 mm in mature worms. In complete worms, the smallest female observed to carry oocytes was 64 mm long and 2.8 mm wide (10th chaetiger); the smallest male with sperm in the coelom was 61 mm long and the width of 10th chaetiger was 2.7 mm.

The first chaetiger with gametes was always located after the branchial region. Branchiae disappear between chaetiger 29 to 40. Oocytes were reported from 35 to 55 (mean 45.84 ± 4.01), and sperm was observed from chaetiger 40 to 55 (44.64 ± 3.32). A low but significant Spearman correlation was observed between the first chaetiger having gametes and total length (rho = 0.393; p<0.001) and between the first chaetiger bearing gametes and the number of branchial chaetigers (rho = 0.383; p<0.001).

The number of chaetigers with gametes ranged in females from 20 to 55 (mean 30.46 ± 6.46), and in males from 20 to 35 (mean 29.38 ± 4.96).

No external morphological differences were noticed between males and females. However, during all year, the chaetigers with sperm were whitish, and in females the yellow oocytes were visible through the body wall in the coelom cavity. Mature males and females were always present during the sampling period, with the latter dominating the population structure through all year (Fig. 20). The male: female sex-ratio ranged from 1:4 and 1:2.

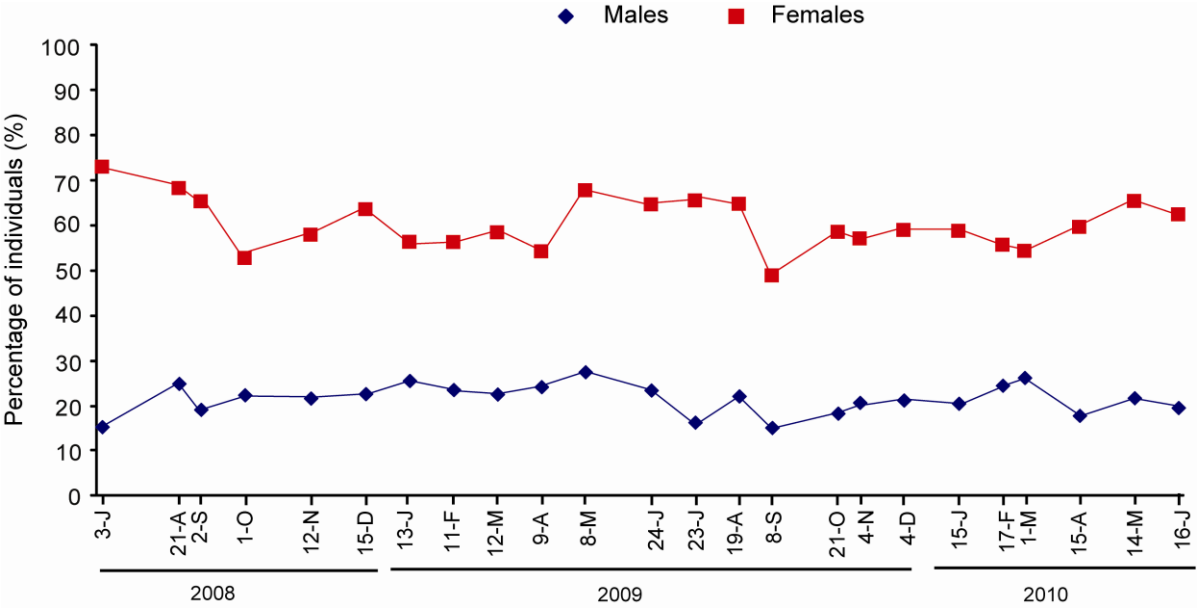

Figure 20 - Monthly percentage of males and females of *D. marocensis* during the study period. Only individuals with gametes were considered.

Oocytes were spherical and yellow. The minimum diameter for the oocytes found in the females coelom cavity was 180 µm and the maximum 740 µm (mean: 497.65 ± 31.38 µm); only a small percentage of the oocytes remaining in the coelom were larger than 620 µm. Immature oocytes present nurse cells attached with two strings, measuring up to 600 to 640 µm long and composed of up to 31 cells (mean 25.2 ± 4.85) with a diameter around 25 µm. Oocytes larger than 550 µm did not have nurse cells attached.

Figure 21 shows the temporal distribution of the proportion of small (<300 µm), medium (300 - 500 µm) and large oocytes (>500 µm). Small oocytes were found during the whole study period, but were proportionally more abundant from March/ April to September/October. Large oocytes were always present and always very abundant. The mean oocyte diameter was higher from November/December to February/March (Fig. 22) corresponding to the period with less small oocytes (Fig. 21). After this period the mean oocyte diameter decrease and the number of small oocytes in the coelom cavity increased (Figs. 20, 21).

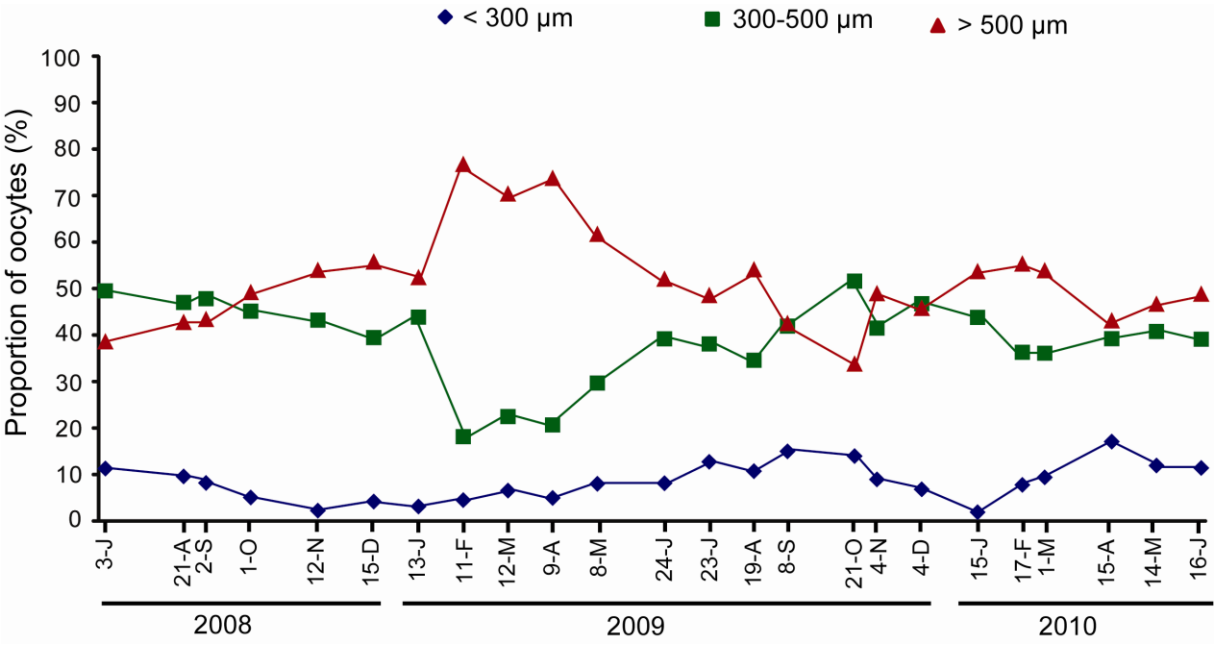

Figure 21 – Size-frequency distribution of the oocytes of *D. marocensis* during the sampling period.

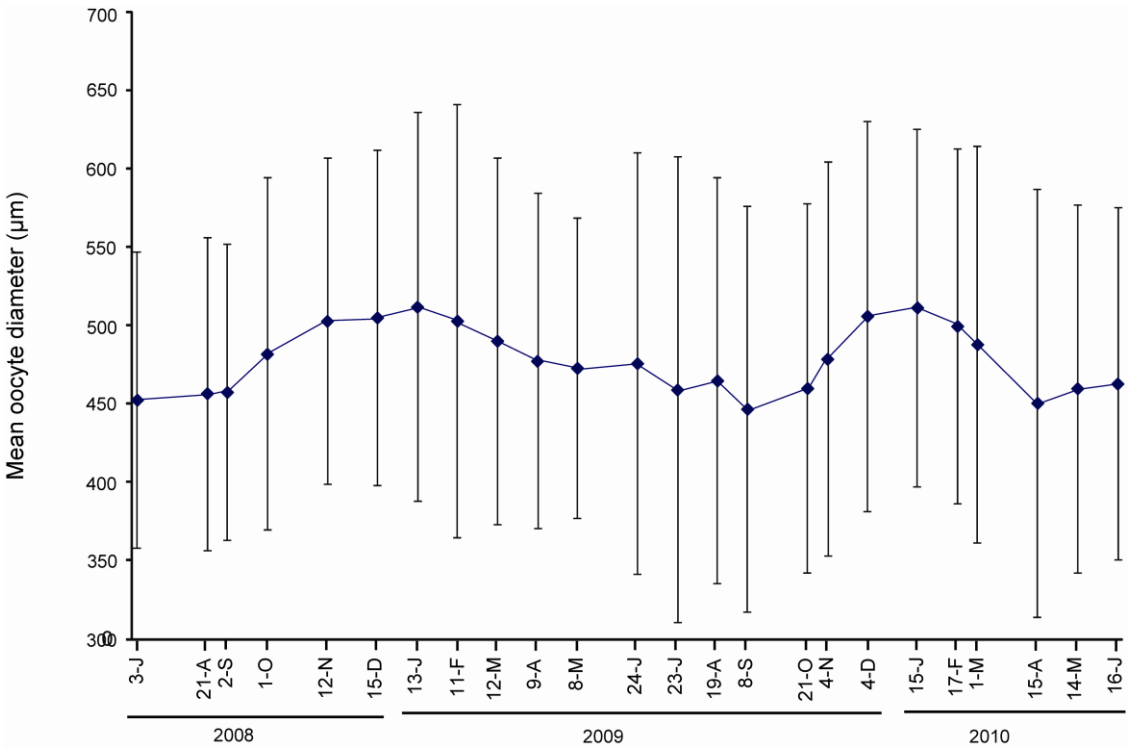

Figure 22 – Monthly, mean oocyte diameter (µm), during the study period. Bars represent standard deviation.

The number of oocytes in the body cavity of whole females varied from 44 to 624 (mean: 276.85 ± 161.54). The evolution of the mean number of oocytes along the study period is shown in fig. 23.

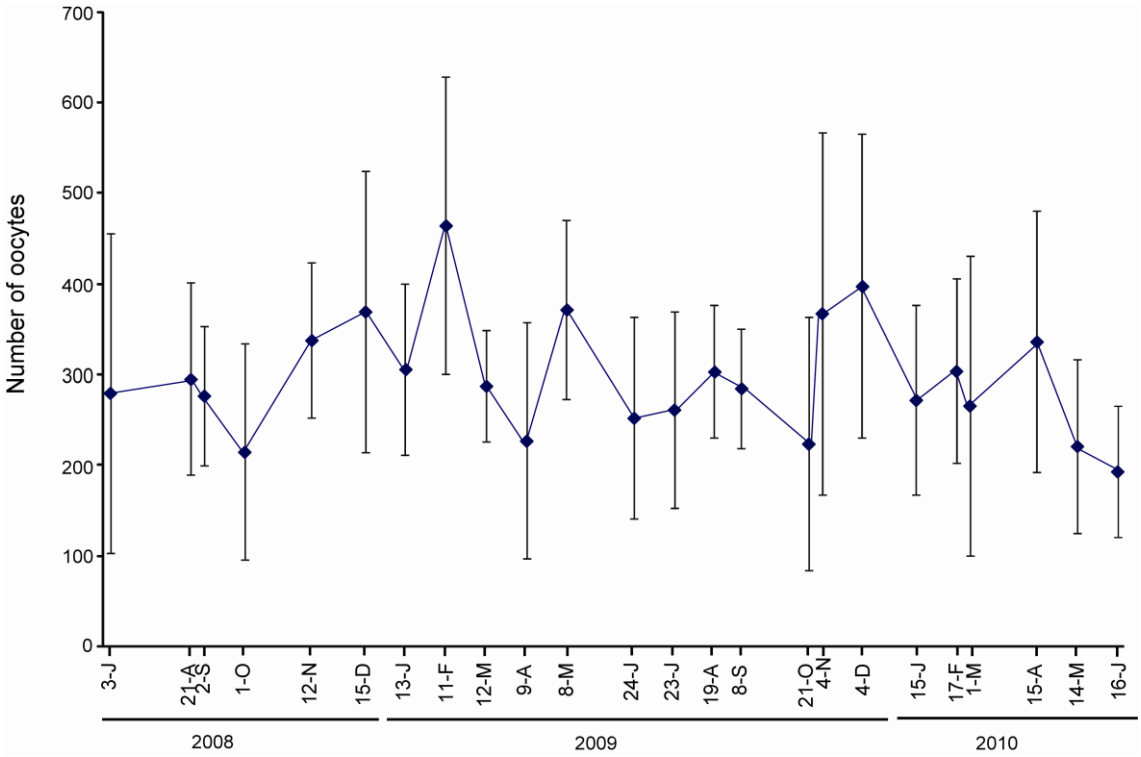

Figure 23 – Monthly mean of the number of oocytes observed in the body cavity of complete females.

The mean oocyte diameter was not significantly correlated with the females total length (rho=0.014; p>0.05), however, the number of chaetigers bearing oocytes was significantly correlated with the specimens size (rho=0.457; p<0.001). The oocytes diameter was also significantly correlated with the number of chaetigers bearing oocytes (rho=0.757; p<0.001).

Diopatra marocensis female adult with eggs or larvae inside their tubes were found during the entire study period. They were located near the adult, on the dorsal side and at the end of the branchial region. These females also contained oocytes in the coelom cavity (41 to 260, in complete specimens). The percentage

of tubes with eggs and larvae ranged between 10% and 50% from all the tubes analysed during the study period (Fig. 24).

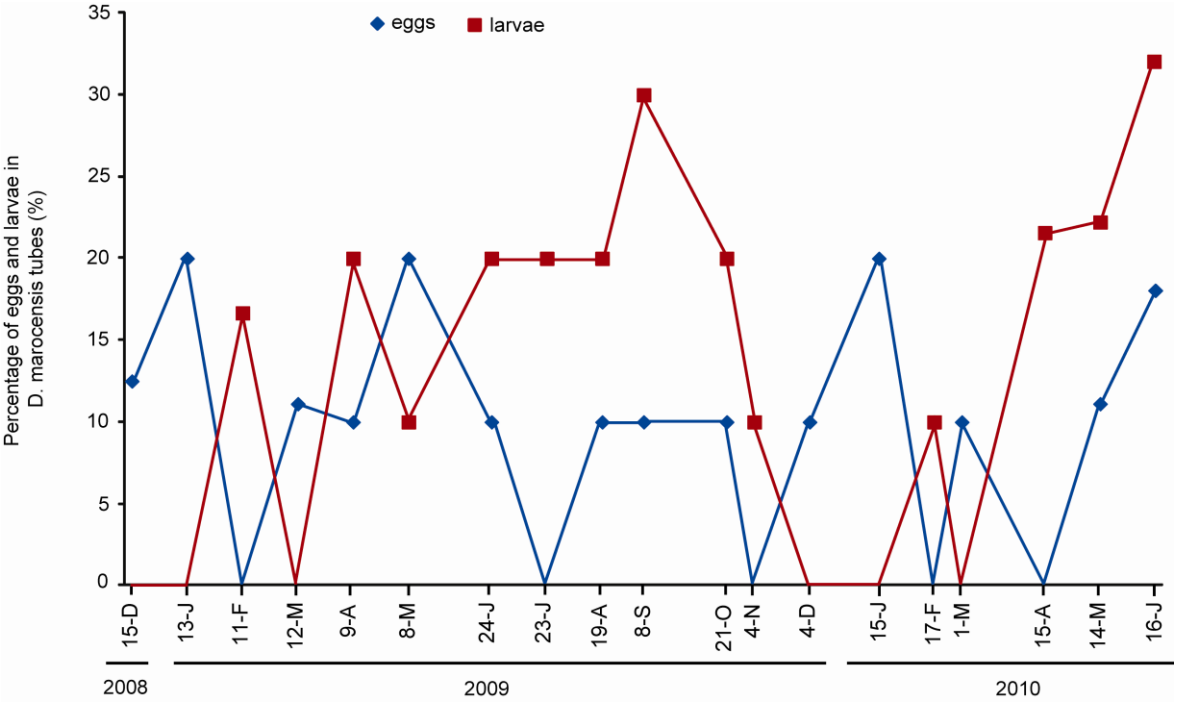

Figure 24 – Monthly percentage of parental tubes bearing eggs or larvae.

The number of eggs in the tubes ranged from 75 to 298 (152.47 ± 66.64) and the larvae from 60 to 194 (102.89 ± 36.12). Most eggs and larvae in a given tube present a very similar size, being at the same developmental stage. Only 2 of the 60 tubes analysed presented two different development phases: one contained 12 eggs and 194 larvae in stage 3; the other had 26 in development stage 2 and 95 in stage 3. The diameter of the eggs found in the tubes varied between 600 and 660 µm (mean 632.90±4.67 µm). The larvae found in different tubes at a given month were in the development stages 1, 2 or 3 (Figs. 24, 25). For example, in June 2010 three tubes were observed containing larvae in a different development phase each (cf. Fig. 25), and also 2 tubes with eggs. The larvae had between 2-3 chaetigers (Fig. 25A), 6-8 chaetigers (Fig.25B) and 23-25 chaetigers (Fig. 25C). These observations suggest that *D. marocensis* is an asynchronous species, as at a given moment the population displays a range of development phases. The main

reproductive period of *D. marocensis,* in Ria de Aveiro*,* was found to be from April to September, however individuals with gametes in the coelom were observed during the whole study period, and larvae and/or eggs in the females tubes, suggesting that this species reproduces during all year.

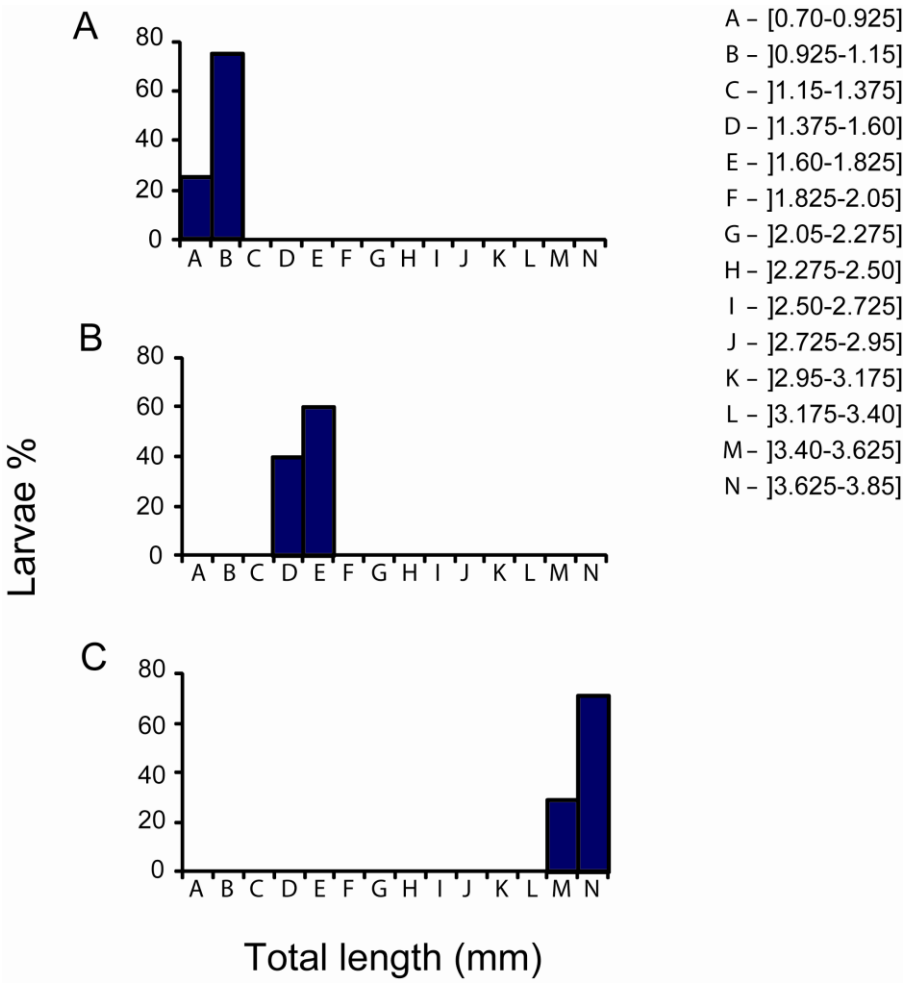

Figure 25 – Size frequency distribution of larvae during the month of June (2010), in three different tubes.

3.3.3 Fertilization *in vitro* of *D. neapolitana*

Table 7 presents the main characteristics of larval development of *D. neapolitana* in this and in others studies (Bhaud and Cazaux, 1987 and Conti and Massa, 1998).

Table 7 – Principal characteristics of larval development of *Diopatra neapolitana* in this study and in comparison with Bhaud and Cazaux (1987) and Conti and Massa (1998). P = Protrocophore, M = Metatrocophore, E = Erpochaeta, J = Juvenile.

	Present study	Bhaud and Cazaux (1987)	Conti and Massa (1998)
P	19h. Shape sub-spherical. Almost completely covered by cilia. Apical tuft. Larvae swimming actively in water column.	24h. Shape sub-spherical to piriform. Length 215 µm. Almost completely covered by cilia. Apical tuft. Larvae swimming in water column.	5h. Larvae swim free in the water column.
M	2 to 3 days. Length 240 to 280 µm. 3 chaetigers. Prostomium ciliated. 2 red eyes.	3 days. Length 240 µm. 3 chaetigers. Prostomium ciliated. 2 red eyes.	24h. larvae present positive phototropism.
M	3 days. Length 300 µm. 4 chaetigers. Loss of apical tuft. 2 red eyes. 4 days. 380 µm. 4 chaetigers. 2 red eyes. Some of them swimming in water column and others on the bottom, with detritus around them (starting the tube construction).	4 days. Length 390 µm. 4 chaetigers. Loss of apical tuft. 2 red eyes. 4 to 5 days. Larvae sink to the bottom and produce mucus were particles will aggregate	3 days. Larvae sink to the bottom. 4 days. Black jaws visible through body cavity. 3 chaetigers. Larvae start to agglutinate diverse detritus.
E	5 to 6 days. 500 µm. 5 chaetigers, 2 red eyes. 5 large buds in prostomium. Rudimentary anal cirri. Black jaws visible through body cavity.	6 days. Length 550 µm. 5 chaetigers. 5 large round antennal buds at the front of the prostomium. Black jaws visible through body cavity, rudimentary anal cirri.	Not described.
J	7 days. Length 540 µm. 5 chaetigers. 2 red eyes. 5 small antennae on the prostomium. 2 anal cirri. Juvenile present positive phototropism.	7 days. Parapodia more developed. 5 antennae. 16 days. Length 1250 µm. 7 chaetigers. Parapodia and antennae more developed.	1 month. 25 chaetigers. First branchia appears in 5th parapodia. 1 month and 20 days. Second branchia appears in 6th parapodia. 3 months. Length 15 mm.

Larval development was followed up to the age of seven days. Seven hours after fertilization the embryo had cilia and swam in the water column, becoming a free-swimming protrochophore larva after 19 hours. The protrochophore larvae were sub-spherical, with an apical tuft and almost completely covered by cilia with a length of 210 µm. Two to three days after fertilization, the metatrocophore larvae had a length between 240 to 280 µm and were segmented in 3 chaetigers (with chaetae); the prostomium was ciliated and had 2 red eyes. After 3 days, the metatrocophore larvae lost the apical tuft, presented 4 chaetigers and a length of 300 µm. On the 4^{th} day, some metatrocophore larvae swam slowly in the water column, and others started to sink to the bottom and began to aggregate detritus around them. At this phase larvae were moved to an aquarium with fine sediment and fresh sea water, in order to allow the larvae to create a wrapping and protecting niche, and later permit the construction of the tube. However tube formation by juveniles was not observed.

Juveniles with five chaetigers, five small antennae on the prostomium and a pair of small anal cirri in the pygidium were observed 7 days after fertilization.

3.4 Discussion

Despite the diversity of *Diopatra* species in marine benthic communities, more than 50 (Budaeva and Fauchald, 2008), the knowledge about their reproductive characteristics is scarce, and the majority acquired from observations of preserved material. The reproductive cycle of *Diopatra neapolitana* is known only from a study on the Mediterranean sea (Dagli et al., 2005) and in the case of *D. marocensis* only its larval development is known (Fadlaoui et al., 1995).

The study of the reproductive biology of *D. neapolitana* and *D. marocensis* showed that both species contained gametes in the coelom during all months of the year. However, *D. neapolitana* had the highest proportion of individuals with gametes from May to August, and *D. marocensis* presented a high percentage of individuals with gametes during all months. Dagli et al. (2005) in Izmir Bay, Turkey, observed also individuals of *D. neapolitana* with gametes all year round, except in January. For all months females of *D. marocensis* dominated the population, with

a sex ratio male: female varying between 1:4 and 1:2, otherwise, *D. neapolitana* male: female sex ratio was about 1:1 along all period study.

Conti et al. (2005) described that mature sperm of *D. neapolitana* had a long tail attached to a spherical, short, and rounded head, which is similar to our findings for sperm from May to August. The sperm also had the highest mobility during this period. These results indicate that the beginning of gametogenesis should be in March/April, the spawning period from May to August, and the gametogenic inactivity from November to February.

Both species cohabit in Ria de Aveiro but they have different reproductive patterns: *D. neapolitana* is a broadcast spawning with a free-swimming larval stage and *D. marocensis* breeds in the parental tube. According to this, some differences on reproductive biology are expected.

The number of oocytes in females' body cavity of *D. neapolitana* is on the order of the thousands and it was higher between May and August, decreasing, however, during this period. In October and December it was noticed that the number of oocytes found in body cavity of females was similar, suggesting that no oocytes were released during this period. A number of small oocytes (<140 μm) was present in almost every month, showing a peak in March and April, and then decreasing until September. The absence of small oocytes between November and January indicates that the females were not producing oocytes. The large oocytes probably were not expelled during spawning and remained in the coelom cavity. The decrease of small oocytes was paralleled by an increase in the number of larger oocytes.

In *D. marocensis* a high percentage of individuals with gametes in the coelom were found in all months. Very few immature and juvenile individuals were also found, throughout all the sampling period. Females bearing eggs and larvae in the tube were present during all the year but in higher numbers and with more eggs and larvae between April and October. This indicates that spawning should occur from March-April to September, being this the main reproductive period of the species. This is corroborated by the mean number of oocytes in the coelom of

entire females, lower from June until October, suggesting that spawning had occurred.

The oocyte diameter of *D. neapolitana* varied between 40 to 240 µm, with a mean of 164.4 ± 40.8 µm. Oocytes should be released from the coelom into the water column with a diameter of about 200 µm (Dagli et al., 2005). In fact, in the present study only a small percentage of the oocytes remaining in the coelom had a larger diameter. This is in agreement with our observations of the artificial fertilization, and with Bhaud and Cazaux's (1987) results, as the fertilized eggs had a diameter between 210-215 µm.

Contrasting with *D. neapolitana*, the oocyte diameter in the coelom of *D. marocensis* females ranged from 180 to 740 µm, with mean 482.91 ± 121.98 µm. When oocytes are larger, they occupy more chaetigers ($rho=0.757$; $p<0.001$). Fadlaoui et al. (1995) referred that mature eggs had a diameter of about 600 µm, and in our study, the eggs found in the tubes presented a mean diameter of 632.90±4.67 µm and only a small percentage of the oocytes in the coelom had a higher diameter. The first larval development stage had about 700 µm, as observed by Fadlaoui et al. (1995).

Nurse cells were observed in immature oocytes of both species. In *D. neapolitana* females, they had a diameter equal or less to 160 µm, as reported by Dagli et al. (2005) and reached up to 39 cells, 230 µm long, and 12 µm wide, which is larger than observed by Dagli et al. (2005). In *D. marocensis*, nurse cells were longer, measuring 600 to 640 µm long and are composed up to 31 cells with 25 µm wide.

In *D. neapolitana*, the first chaetiger with gametes ranged in females from the 35^{th} to the 70^{th} and in males from the 50^{th} to 70^{th}, usually after the chaetigers with branchiae. These results are similar to what was obtained by Dagli et al. (2005), for females, but quite different for males. These author reported the appearance of oocytes from chaetiger 35 to 78 (mean 55 ± 0.9) and of sperm from chaetiger 32 to 85 (mean 51 ± 0.9). In agreement with those authors, our study also showed the absence of a significant correlation between the first chaetigers with oocytes and

the width of the 10th chaetiger, chosen as a measure of the total length of the specimens.

Gametes of *D. marocensis* were also observed in chaetigers after branchiae. The appearance of gametes in chaetigers when branchiae had already disappeared or are almost finishing was observed for others *Diopatra* species (cf. Table 8). In *D. lilliputiana* sperm was observed from chaetiger 35 until the end of the body, and branchiae finished from chaetigers 26-37; for *D. hanleyi* oocytes appear from chaetiger 50 and branchiae disappear from chaetigers 50-60 (Paxton, 1993).

In the present study, the smallest mature *D. neapolitana* male and female were 139.2 mm and 149.7 mm long, respectively. These values are higher than those obtained by Dagli et al. (2005) who reported minimum length in females of 125 and in males of 110 mm. In their study, the largest entire worm had a length of 347 mm. During our collecting period 46 complete individuals were harvested with a total length between 24 mm and 725 mm. Only specimens to about 600 mm long were referred in the literature.

The artificial fertilization experiment conducted in this study concluded that *D. neapolitana* has a free-swimming larval stage and this is in agreement with the conclusions of Dagli et al. (2005). In our fertilization experiment, free-swimming protrocophore larvae were obtained 19h after fertilization, at 22ºC. In Conti and Massa (1998), this phase appeared 5 hours after fertilization (25-32ºC) and Bhaud and Cazaux (1987) only observed the protrochophore larva 24 hours after fertilization. In Conti and Massa (1998) the larvae developed faster than in our study and that of Bhaud and Cazaux (1987). Morphologically, the results of our experiment were similar to Bhaud and Cazaux's (1987) larval description, but the time of development was different, as in our study larvae developed faster (about 1 day) until metatrocophore phase.

These differences could be explained by the temperature, 22 ºC in our case, and 25-32 ºC in Conti and Massa (1998) experience, although temperature is never clearly mentioned in Bhaud and Cazaux (1987). The number of larvae or the

size of the containers could influence its development, but we do not have information about these features.

Larvae *of D. neapolitana* observed in our experiments and in Bhaud and Cazaux (1987) were morphologically different to those described by Choe (1960) as being of *D. neapolitana*. This supports Paxton's (1993) suggestion that the species mentioned by Choe (1960) was not *D. neapolitana* but a different species (Paxton, 1993).

Larval development has also been studied in other *Diopatra* species, namely *D. cuprea* (Allen, 1959), that has a developmental pattern similar to *D. neapolitana*, and *D. marocensis* (Fadlaoui et al., 1995) that breeds in the parental tube. The first larval stage observed in this study for *D. neapolitana*, the protrochophore, was similar to that described for *D. cuprea* (Allen, 1959) and *D. marocensis* (Fadlaoui et al., 1995). This stage is characterized in the three species by the presence of the apical tuft and ciliation around the body. *D. neapolitana* and *D. cuprea* are active swimmers and had red eye spots, during the initial development stages. *D. cuprea* starts to settle to the bottom 3 days after fertilization, with 4 chaetigers, producing mucous to build the tube (Allen, 1959). This was similar to what was observed in this study for *D. neapolitana*. Five antennae and anal cirri were observed at the 5th-chaetiger stage in *D. cuprea* and *D. neapolitana*, and at the 6th-chaetiger for *D. marocensis*. In *D. marocensis*, the ceratophores appear at the 12th-chaetiger stage, in *D. cuprea* at the 5th-chaetiger, with 1 to 2 rings, whereas in *D. n*eapolitana the ceratophores still had no rings at the 50th-chaetiger stage (Conti and Massa, 1998). According to these authors, the first branchiae appear at the 25th-chaetiger stage on the 5th chaetiger in *D. neapolitana*, and at the 18-20th chaetiger stage in *D. marocensis*, also on the 5th chaetiger (Fadlaoui et al., 1995).

According to the classification system proposed by Fadlaoui et al*.* (1995) to *D. marocensis*, larvae in all developmental stages were found in the parental tubes. The longest presented 25 chaetigers and 3850 µm total length.

With the exception of two tubes, all the larvae inside a given tube were all at the same developmental stage, suggesting they originate in the same spawn.

Table 8 – Summary of reproductive characteristics studied for the genus *Diopatra*: I – broadcast spawning, free-swimming stage, planktonic larvae with lecithotrophic development; II – egg mass attached to parental tube, direct development; III – brooding in parental tube, direct development; ? – Unknown.

Species	Type of development	Max. width 10th chaetiger, adult (mm)	Brood size	Max. eggs diameter (µm)	Brood care	1st chaetiger with gametes	End of branchiae (chaetiger)	Habitat	Location	Reference
D. cuprea Bosc, 1802	I	10.0	?	220	Eggs released in jelly mass which readily dissolves in seawater; reared for 21 days in laboratory.	?	?	-	NE. U.S.A	Allen 1959
D. neapolitana Delle Chiaje, 1841	I	13.0	thousands	200	Mature gametes in body cavity; Artificial fertilization; reared in laboratory up juvenile stage; spawning since May to August.	35-70	56-70	Intertidal	Aveiro, Portugal	Pires et al. 2011
D.? *sugokai* Izuka, 1907 as *D. neapolitana* Delle Chiaje, 1841	I	10.0	?	200	Eggs in jelly mass attached to the tube opening. Artificial fertilization; larvae reared in the laboratory	?	?	Intertidal	Japan	Choe, 1960
D. aciculata Knox and Cameron, 1971	I	11.5	thousands	200-230	Oocytes or mature sperm in body cavity in November, January and March. No eggs or larvae were found in any tubes or attached to them.	?	10-60	Intertidal to 66 m	S. Australia	Paxton 1993
D. amboinensis Audouin and Milne Edwards, 1833	I	6.0	thousands	200-230	Gametes in body cavity from March to August. No eggs or larvae were found in any tubes or attached to them.	?	24-71	Intertidal to 25 m	N. Australia	Paxton 1993

(To be continued.)

Table 8 – (continued.)

Species	Type of development	Max. width 10th chaetiger, adult (mm)	Brood size	Max. eggs diameter (µm)	Brood care	1st chaetiger with gametes	End of branchiae (chaetiger)	Habitat	Location	Reference
D. amboinensis Audouin and Milne Edwards, 1833	I	6.0	thousands	200-230	Gametes in body cavity from March to August. No eggs or larvae were found in any tubes or attached to them.	?	24-71	Intertidal to 25 m	N. Australia	Paxton 1993
D. dentata Kinberg, 1865	I	7.0	thousands	240-260	Oocytes or mature sperm in body cavity from October to February. No eggs or larvae were found in any tubes or attached to them.	?	28-62	Intertidal to 30 m	E. Australia	Paxton 1993
D. dexiognatha Paxton & Bailey-Brock, 1986	I?	1.8	?	180	Oocytes in body cavity	?	?	Intertidal	Hawaii, U.S.A.	Paxton and Bailey-Brock, 1986
D. hanleyi Paxton, 1993	I?	3.0	thousands	170	Oocytes in body cavity in February	50	50-60	Intertidal	N. Australia	Paxton 1993
D. ornata Moore, 1911	I?	3.0	900	235	Oocytes in body cavity	?	?	-	S.W. USA	Fauchald 1983
D. micrura	I?	4.5	?	180-200	Oocytes and sperm in body cavity	?	32-55	Intertidal to 60 m	Aveiro	Present study
D. sp. nov. as *D. cuprea*	I?	2.0	3200	200	Oocytes in body cavity	?	?	-	S.E. USA	Fauchald 1983

To be continued.

Table 8 – (continued.)

Species	Type of development	Max. width 10th chaetiger, adult (mm)	Brood size	Max. eggs diameter (µm)	Brood care	1st chaetiger with gametes	End of branchiae (chaetiger)	Habitat	Location	Reference
D. sp as *D.* sp.nov., from Arcachon	I?	7.5	?	200-220	Oocytes and sperm in body cavity	?	?	Intertidal	Arcachon, France	Present study
D. albimandibulata Paxton, 1993	II	3.5	250	280	Larvae develop in globular small mucous egg sacs (3.0 to 4.5 mm in diameter) attached to the distal end of parental tube	?	30-44	5-18.5 m	E. Australia	Paxton 1993
D. maculata Paxton, 1993	II	9.0	thousands	350	Eggs and 3 to 4-chaetiger larvae enclosed in a mucous matrix on outside of distal end of tube	?	54-68	Intertidal	W. Australia	Paxton 1993
D. sp as D. neapolitana D. Chiaje, 1841	II	10.0	?	?	Yellow eggs in a brown and irregular gelatinous mass (1cm^2 size) on distal end of tube	?	?	Intertidal	Italy	Lo Bianco 1898
D. gigova Paxton, 1993	III	3.5	9	1400	Eggs in a mucous sac attached to the tube 1.5 cm from its distal end	?	30-42	Intertidal	W. Australia	Paxton 1993
D. lilliputiana	III	1.3	40	700	Eggs and larvae develop in mucous sac (about 6mm long for eggs and 7mm for larvae) inside parental tube at least to the 15th chaetiger	35	26-37	Intertidal	W. Australia	Paxton, 1993

To be continued.

Table 8 – (continued.)

Species	Type of development	Max. width 10th chaetiger, adult (mm)	Brood size	Max. eggs diameter (µm)	Brood care	1st chaetiger with gametes	End of branchiae (chaetiger)	Habitat	Location	Reference
D. marocensis Paxton *et al*., 1995	III	5.7	298	600-620	Larvae up to 28-34-chaetiger develop inside the female tube	35-55	29-40	Intertidal; 16 – 40 m	S. Morocco; Western Portugal (Ria de Aveiro)	Fadlaoui et al. 1995; Present study
D.? *marocensis* Paxton et al., 1995 as *D. cuprea* Bosc, 1802	III	?	?	?	Eggs and up 11-chaetiger larvae in parental tube	?	?	-	Madeira	Monro 1924
D. tuberculantennata Budaeva & Fauchald, 2008	III	2.0	5	?	Five 28-chaetiger larvae found in the parental tube	?	14-37	0-2 m	Caribbean Sea, Belize, West of Dangriga	Budaeva & Fauchald 2008
D. variabilis Southern, 1921	III	3.0	30-50	600	Eggs and larvae of various stages develop in jelly membrane attached to the inside walls of the parental tube; reared to 15-chaetiger stage in laboratory	?	?	Shallow brackish water	India	Krishnan 1936; Krishnamoorthi, 1963
D. sp.nov. as *D. amboinensis* Aud. & M. Edw., 1833	III	3.0	?	?	Eggs singly attached inside parental tubes; Larvae up to 6-chaetiger; juveniles build own tube on outside of parental one.	?	24-71	-	Indonesia as Java	Lieber 1931,
D. sp. as *D.* sp. nov.,	III	?	?	"gigantic"	Larvae in the parental tube	?	?	-	Indonesia as Sumatra	Lieber 1931,

Diopatra that are broadcast spawners are the largest species in the group with the width of 10th chaetiger ranging from 6.0 to 13.0 mm. Their eggs are small, with a diameter ranging from 200 to 260 µm, but numerous, in the thousands per female. Individuals that attach their eggs and larvae to the parental tube are smaller, with the width of 10th chaetiger ranging from 3.5 to 10.0 mm, while the eggs are larger, with diameter from 280 to 350 µm, and varying in number from 250 to thousands per female.

A single *Diopatra* species was recognized as developing directly in a cocoon, and only a few juveniles were observed, so the information concerning adult size, brood size and egg diameter is not available for this type of development. No more *Diopatra* species were observed having this type of development. Paxton (1993) considered this observation doubtful, suggesting that the supposed cocoon may be the remains of a broken tube. Before confirmation is obtained, we also suggest that this should not be considered a valid development mode for *Diopatra* species.

Adult specimens brooding in the parental tube are the smallest of the group, with width of 10th chaetiger up to 5.7 mm, but the eggs are the largest: 600 to 1,400 µm in diameter, and fewer, with just up to 300 per female. The majority of the species in this group have their eggs and larvae enclosed in a mucous matrix. Krishnamoorthi (1963) in a detailed study of the effect of hypotonic and hypertonic media on eggs of *D. variabilis*, concluded that the mucus might provide a barrier against varying environmental conditions for the healthy development of the eggs. *D. marocensis* is the largest known *Diopatra* species with this type of development. It is also the species which lays the largest number of eggs and larvae inside the parental tube.

Females of *D. marocensis* that contained larvae and eggs in their tubes had a low number of oocytes in the coelom. Some *D. neapolitana* females had also a small portion of oocytes in coelom since November to February. *D. lilliputiana* individuals displayed also a low number of oocytes in the coelom (Paxton, 1993). Probably these gametes will be available as reserve material for the following gamete production, as occurs with another Onuphidae species, namely *D. neapolitana* (Pires et al., 2011) and *Marphysa sanguinea* (Prevedelli et al., 2007).

This was also reported in others iteroparous marine organisms and has been realized as an aspect of a suitability response trade-off “present reproduction” in opposition to “future reproduction” (Olive et al., 1997).

For *D. micrura*, a new species found in Ria de Aveiro (Pires et al., 2010) a single female with oocytes in the coelom was observed. They had a diameter between 140 and 200 µm (mean=174.29±19.02). Considering the observed developmental patterns (cf. table 8), and that nor larvae or eggs were observed in the tubes, brooding inside them seems doubtful. So we assumed that this species could be a free spawning. Similarly, the oocytes found in coelom of *Diopatra sp* from Arcachon (Pires et al., 2010) were relatively small, ranging from 120 to 220 µm (in maximum 200-220 µm), and the species could also be a free spawning.

Revising all information for the genus *Diopatra* (cf. Table 8), the majority of the species either brood in the parental tube with direct development (8 species) or are broadcast spawners, with a free-swimming stage (6 species). The developmental mode for *D. dexiognatha*, *D. hanleyi*, *D. ornata* and *D. sp.* nov. as *D. cuprea,* listed in table 1, is unknown. However, they all have relatively small eggs, varying from 170 to 235 µm, and considering the observed developmental modes it seems improbable that they brood in the tube. So it seems reasonable to assume that they could be free spawning; more studies are needed to confirm these suggestions.

During the study period, some individuals of both species were regenerating the anterior end of the body. The majority of *D. neapolitana* individuals that were regenerating the anterior end had no gametes in the coelom, except for some females that contained small oocytes. It is thus possible that the individuals in regeneration concentrate all their energy in this process. Some studies revealed that tissue loss has a negative effect upon growth and reproduction (Zajac, 1985, 1995; Irlandi and Mehlich, 1996; Nilsson and Skold, 1996; Hentschel and Harper, 2006). A study with the spionid polychaete *Polydora ligni* showed that the regeneration costs in terms of fecundity loss were estimated to range between 10% and 29% for palp regeneration, and between 49% and 80% for posterior regeneration (Zajac, 1985). This author determined individual fecundity in

regenerating females, by counting the number of eggs or larvae deposited within a string of capsules, inside the tube. Females continued to reproduce while regenerating lost palps or posterior segments, but posterior segment loss also increased brood development time (Zajac, 1985). A different scenario was found for *D. marocensis*, for which the majority of the specimens that were regenerating (about 68%) had oocytes in the coelom cavity.

Chapter 4

Regenerative ability of the polychaete *Diopatra neapolitana*

4.1 Introduction

Among the polychaetes, the regeneration ability differs from species to species and almost all can regenerate appendages and the posterior end of the body (Brusca and Brusca, 1990; Bely, 2006). *Diopatra* species are also able to regenerate anterior segments (Bely, 2006).

In Ria de Aveiro, field collected *D. neapolitana* and *D. marocensis* specimens were found regenerating, mainly the anterior segments, indicating that only rarely the species autotomizes the posterior part of the body or has it susceptible to predation (Pires et al., 2011 and unpublished data).

As referred in previous chapters *D. neapolitana* is a tubicolous polychaete that can grow up to 70 cm long and is harvested as fresh bait, but often only the anterior part (10-15 cm) is collected by bait diggers (Cunha et al., 2005).

In this study we evaluate the regenerative ability of *D. neapolitana* under laboratory conditions following nine experimental amputation levels, in order to understand the capacity of the species to regenerate body damage caused by natural predation or bait digging activities.

4.2 Materials and Methods

4.2.1. Sampling

Specimens of *D. neapolitana* were collected in intertidal areas in Ria de Aveiro, inside their tubes, with a shovel. In the laboratory, the specimens were pushed out from the tube and kept in aquaria with sand for at least two months for acclimation, at 22 ºC and at salinity in the range 30-35. *D. neapolitana* adults are very long, being nearly impossible to collect whole individuals, so this acclimation period is important to allow reestablishment and regeneration of the posterior damaged portion. Field-collected specimens which were already regenerating were not used in the experiment. These specimens could be distinguished by the lighter colour and/or the narrower regenerating chaetigers, when compared to the rest of the body.

4.2.2 Regeneration in *D. neapolitana*

4.2.2.1 Laboratory experiments

The regeneration from nine experimental amputation levels was conducted under laboratory conditions: before the beginning of the branchiae (chaetiger 3 or 4); in the branchial region, at chaetiger 10, 15, 20, 25, 30, 35 and 40, and after the end of the branchiae (chaetigers 45-55). Sixteen individuals were used for each treatment, collected from the laboratory culture, a total of 144 specimens for the whole experiment. The specimens were anesthetized with a solution of 4% $MgCl_2.6H_2O$, pushed out from their tubes and were amputated at each level with a scalpel under a stereomicroscope. The posterior body parts were kept in their tubes, to simulate amputation from predation or bait digging, and the anterior ends were allowed to burrow in the sand.

After recovering from the anaesthetic, each worm was placed in a PVC container (17 cm height x 11 cm diameter), filled with sand and inside a large aquarium, with fresh sea water, at 22 ºC and with salinity in the range 30-35.

Both the anterior and the posterior body parts from each individual were maintained in the aquarium, in the same container, in order to notice which or if both parts were able to regenerate. Individuals that died during the experiment where removed from the containers. *S*pecimens were fed with frozen cockles and fish commercial dried food, every 2 to 3 days. The excess food was never allowed to accumulate in the test containers.

During the study period, specimens that were regenerating the anterior end were observed when they emerged from their tubes to feed. In order to observe the worms under a stereomicroscope, the tubes were removed from the sediment and placed in seawater, and then the specimens were anesthetized and partially removed from their tubes (the regenerating portion and a few more segments). The number of regenerating segments was counted per worm. After observation, all specimens were gently pushed back into their tubes, allowed to recover from the anaesthetic in fresh seawater and reinstalled in the experimental container. The experiment was considered finished when a complete regeneration of the amputated body portions was observed, i.e., when no difference could be noticed

between the width of the older and the new regenerated segments. This took from 50 to 70 days, depending on the area of the experimental amputation.

4.2.2.2. Field specimens

Field collected specimens of *D. neapolitana* and *D. marocensis* during the study of the reproductive biology (chapter 3), showing regeneration were observed under a stereomicroscope. The number of regenerating chaetigers was counted in the specimens that were regenerating the anterior end; the first chaetiger with subacicular hooks was registered as well as the number of whorls of the branchiae at chaetigers 5 to 10, where they reach the maximum number (Rodrigues et al., 2009).

4.3 Results

4.3.1 Laboratory experiments

Figure 26 shows various regeneration stages of anterior and posterior segments. Table 9 and Figure 27 indicate the percent survival and other statistics for the various experimental amputation levels studied. During the initial 4 to 6 days following amputation, it was observed that the anterior cut regions were healing. By days 6 to 10 a protuberance of tissue in the anterior end was observed (Fig. 26A). Posterior cut regions healed in 4 to 10 days, and a protuberance of tissue was observed on the posterior end from day 6 to 12. After 10 to 12 days, the individuals had regenerated the anterior end, but to a much smaller size than the original (Fig. 26B). Posterior regenerated chaetigers were observed between days 12 and 18, but also much narrower than the original (Fig. 26C).

It was noticed that specimens regenerating the anterior end rarely expose themselves outside the tube, before the missing portion is actually regenerating.

The anterior portion of the specimens amputated up to chaetiger 15 did not survive. In the specimens amputated at chaetiger 20, neither portion survived. For the specimens amputated at chaetiger 25 and beyond, only the anterior portions survived and regenerated. In all experimental amputation levels, only the posterior

portion was left inside the original tube. The anterior portion was placed in the sand and it was observed that for the specimens amputated at chaetigers 25 and beyond, this anterior portion built a new tube in less than 24 hours, by aggregating sand particles to mucous.

The regenerated anterior and posterior ends were lighter in colour than the original (Figs. 26D; 26E), and the posterior regenerated segments were almost transparent, showing the blood vessels (Fig. 26E). A detailed description of the results obtained at the various amputation levels follows (see Table 9 for summary statistics).

4.3.1.1. Amputation up to chaetiger 15

Up to chaetiger 15, the amputated anterior portion was unable to survive. The percent survival of the specimens that were amputated before the beginning of the branchiae and in the branchial region, at chaetiger 10 and 15, was, respectively, 87.5%, 75% and 50%. Such specimens survived the experiment and regenerated the missing anterior part of the body (Fig. 27, Table 9).

By days 4 to 6, the cut region was healed. After 6 to 8 days, a small reddish protuberance was observed (Fig. 26A). By day 10 to 12, the specimens amputated before the beginning of the branchiae had regenerated small heads and chaetigers (3 or 4, the same number that was cut), peristomium and prostomium with salient nuchal grooves and ceratophores with 6 rings, but the peristomial cirri were still not observed. At this stage, the anterior regenerated portion was much narrower than the original. At day 12, a small anterior end had regenerated in specimens amputated at chaetiger 10 and 15, with prostomium, peristomium and with 8 to 10 chaetigers in the specimens amputated at chaetiger 10, and with 9 to 13 chaetigers in the specimens amputated at chaetiger 15. The ceratophores had 3 to 4 rings and salient nuchal grooves. The width of the regenerated segments was only 15% to 30% the width of the older segments (Fig. 26 B).

After 18 to 20 days, the peristomial cirri already had the same length as the peristomium (their normal size is twice the length of the peristomium) and the ceratophores presented 12 rings. The regenerated portion in individuals

amputated at chaetiger 10 and 15 was 40 to 50% wide, compared to the original. The specimens had the same number of chaetigers as in day 12 and chaetigers had small branchiae, with 3 to 4 whorls, beginning at chaetiger 4 or 5.

Full regeneration (when the new regenerated chaetigers had the same width as the older chaetigers) was observed by day 50 in the specimens amputated before the beginning of the branchiae, between day 50 to 60 in the individuals amputated at chaetiger 10 and between days 60 to 70 in the individuals amputated at chaetiger 15 (Table 9). The regenerated portion persisted lighter than the original segments (Fig. 26D). At this stage, the branchiae from the regenerated chaetigers 6 to 8 of the individuals amputated at chaetiger 15, had a maximum of 10 whorls. After 75 to 80 days, these branchiae presented 14 to 18 whorls, the same number of a normal adult. Subacicular hooks were present from chaetiger 17 onwards in regenerated specimens, whereas in normal adults they are present from chaetiger 19 onwards. This indicates that the individuals regenerate fewer segments than those initially amputated, except if the amputation is made before the branchial region.

4.3.1.2. Amputation at chaetiger 20, mid branchial region

The individuals that were amputated at mid branchial region, at chaetiger 20, did not survive. None of the parts was able to regenerate (Fig. 27).

4.3.1.3 Amputation at and beyond chaetiger 25

The posterior portions of all the specimens amputated at and beyond chaetiger 25 did not survive. The percent survival of the specimens that were amputated at chaetiger 25, 30, 35, 40 and after the end of the branchiae, was respectively 50%, 56.25%, 68.75%, 81.25% and 100% (Fig. 27). The anterior portions of those specimens survived and were able to regenerate a posterior end (Fig. 27, Table 9)

Table 9 - Regeneration time, number of regenerated chaetigers and percent survival for *D. neapolitana* during the study period (n = 16 for each amputation level).

Section regenerating	Chaetigers amputated	Healing (days)	Appearance protuberance (days)	Section regenerate (days)	Appearance of branchiae (days)	Full regeneration (days)	Number of regenerated chaetigers	Survival (%)
Anterior end	3-4	6	8	10 - 12	-	50	3-4	87.5
	10	6	8	12	18 - 20	50 - 60	8-10	75
	15	4	6	12	18 - 20	60 - 70	9-13	50
	20	-	-	-	-	-	-	0
Posterior end	25	6	10-12	18	30 - 40	60-70	40-80	50
	30	4	6	12	30	60 - 70	40-75	56.25
	35	4	6	12	30	60 - 70	40-80	68.75
	40	4	6	12	30	60	40-85	81.25
	Branchiae end (45-56)	4	6	12	-	50	44-90	100

day 6 to 10, specimens amputated at chaetiger 25 healed the cut region and 12 days after amputation, a small reddish not differentiated protuberance was observed, with rudimentary anal cirri.

Between days 4 and 6, the individuals amputated at chaetiger 30 and 35 healed the cut region and by day 6 to 12 a small protuberance was also observed.

By day 6, in the individuals amputated at chaetiger 40 and after the end of the branchiae, a small undifferentiated protuberance was observed, with two small bulges on terminal end, which will become the anal cirri. This was 25% - 30% as wide as the rest of the body.

After 12 days, the individuals amputated after the end of branchiae had 25 to 35 regenerated chaetigers that were about 30 to 40% as wide as the older chaetigers.

After 20 days, all the individuals presented very small chaetigers regenerating and anal cirri. The segments were 40% as wide as the rest of the body, except in specimens amputated after the end of branchiae, where the regenerated portion was 50 to 60% as wide as the original chaetigers. The chaetigers were well differentiated with visible chaetae. By this day, the specimens amputated at chaetiger 40 had regenerated 25 to 50 chaetigers, and specimens amputated after the end of branchiae had 25 to 52 regenerated chaetigers.

Branchiae were present after 30 days in the specimens amputated at chaetiger 25, 30, 35 and 40, but with fewer whorls (1 – 3) and the chaetigers were about 60% as wide as the originals.

40 days after amputation, the regenerated chaetigers of the specimens amputated after the end of the branchiae were 80% as wide as the original and 44 to 90 chaetigers had regenerated.

At day 50, the section regenerated in specimens amputated at chaetiger 25, 30, 35 and 40 was about 80% of the width of the older segments. By this time, the regenerated chaetigers of the individuals amputated after the end of branchiae recovered their full width.

At day 60, the regenerated segments of the individuals amputated at chaetiger 40 had the same width of the older ones. The regenerated chaetigers of the

individuals amputated at chaetiger 25, 30 and 35 reached the same width as the original from 60 to 70 days after the amputation (Table 9).

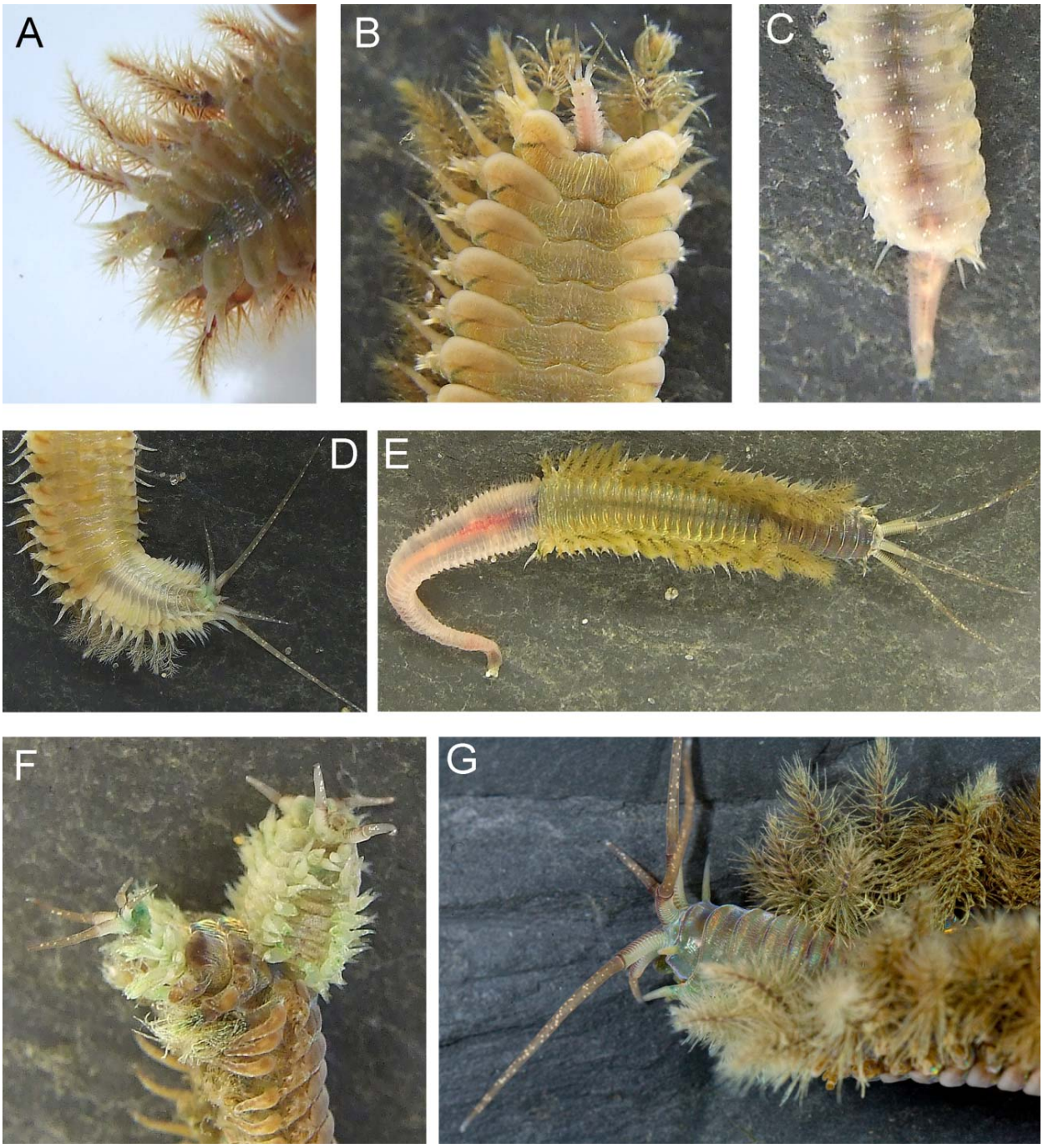

Figure 26 – *D. neapolitana*, anterior and posterior regeneration. A – Specimen regenerating the anterior end, ventral view, 8 days after amputation. A small reddish protuberance is observed at the cut region; B – Specimen regenerating the anterior end, ventral view, 12 days after amputation; C – Specimen regenerating the posterior end, 12 days after amputation; D – Fully regenerated specimen, ventral view, 70 days after amputation; E – Specimen regenerating the posterior end, 70 days after amputation; F – Field-collected specimen regenerating two anterior ends; G – Uninjured specimen.

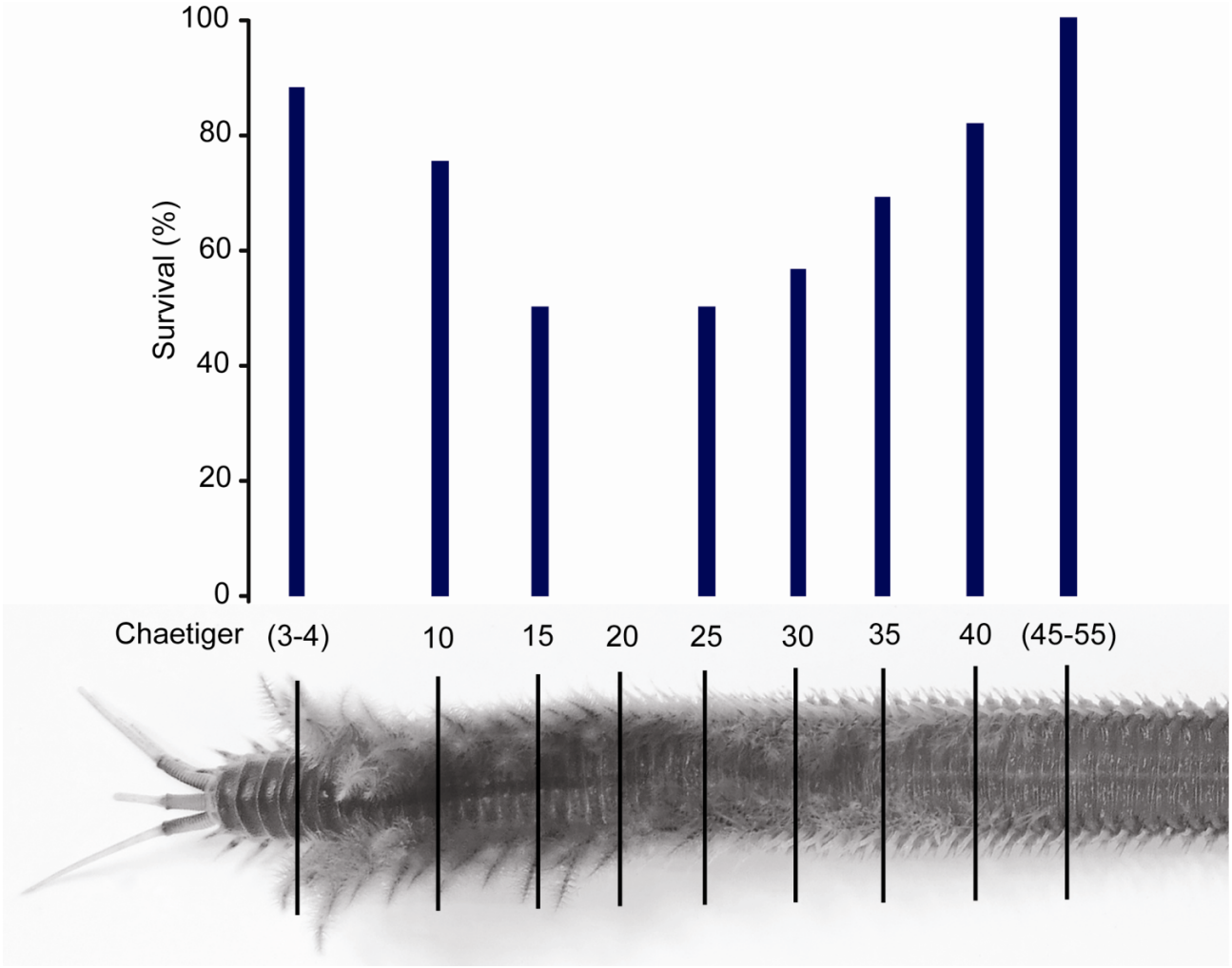

Figure 27 – Survival percentage of *Diopatra neapolitana* specimens at each amputation level.

4.3.2. Field regenerating specimens

Field collected specimens showing regenerating chaetigers were distinguished by having a portion of their body with narrower and/or lighter chaetigers. Figure 28 shows the proportion of specimens regenerating the anterior end along a two year period, for field collected specimens of *D. neapolitana* and *D. marocensis*. The percentage of specimens regenerating the anterior end was determined from all the specimens collected for each month.

4.3.2.1. *Diopatra neapolitana*

It was collected in total 1246 individuals of *D. neapolitana*, of which only 52 were complete, 77 were regenerating anterior end and 6 posterior end.

Field specimens of *D. neapolitana* were found regenerating 4 to 13 chaetigers on the anterior end (mean = 9.0 ± 2.51), plus the prostomium and the peristomium. Some individuals showing just a small protuberance, without differentiated chaetigers, were also observed.

Among the collected specimens, two individuals were found regenerating two anterior portions simultaneously (Fig. 26F). In one specimen, the two heads presented the same size and were regenerating 7 and 5 chaetigers each; in the other specimen, one head was narrower (regenerating 5 chaetigers), than the other (regenerating 6 chaetigers) (cf. Fig. 26F).

The branchiae in fully regenerated chaetigers 5 to 10, presented 6 to 10 whorls (Fig. 26D), whereas uninjured adult specimens had, in the same chaetigers, branchiae with 14 to 18 whorls (Fig. 26G). Despite the regenerated chaetigers had reached their full size, the branchiae were still growing.

Subacicular hooks were observed from chaetiger 17 in the regenerated specimens, when they usually start from chaetiger 19 to 25, showing that fewer chaetigers were regenerated than those lost, confirming the data obtained in the laboratory experiments.

Figure 28A shows the proportion of *D. neapolitana* specimens regenerating the anterior end for two sampling years. The proportion of individuals that were regenerating the anterior end varied between 0 and 17%. This proportion was higher from January to April. Only six individuals were observed regenerating the posterior end (11.5% from the total of complete individuals) and in five sampling occasions, randomly scattered throughout the sampling period. This proportion however may be underestimated given that the posterior end of the majority of the specimens was damaged during sampling.

4.3.2.2. *Diopatra marocensis*

A total of 1722 individuals of *D. marocensis* were collected during the sampling period, of which 633 were complete, 234 were regenerating anterior end and 5 posterior end.

Field collected *D. marocensis* specimens regenerating 4 to 11 chaetigers at the anterior end were obtained (mean = 7.5 ± 1.93), plus the peristomium and the prostomiom. Some individuals only healing the cut region, and others with a small protuberance but without differentiated chaetigers were also observed.

Regenerated specimens had the subacicular hooks from chaetiger 12 to 15, when they usually start from chaetigers 13 to 15, indicating that fewer chaetigers can be regenerated than those lost.

The branchiae in fully regenerated chaetigers 6 to 9, presented 4 to 6 whorls, while uninjured adult specimens presented in the same chaetigers branchiae with 6 to 9 whorls.

The proportion of individuals regenerating anterior end along a two year survey is shown in Fig. 28B. The proportion of individuals regenerating the anterior end varied from 3.7 to 36.5% of the sampled specimens. This proportion was higher from June to September and also higher than that observed for *D. neapolitana* (Fig. 28A). Just five individuals regenerating the posterior end were observed scattered throughout the sampling period (0.80% from the total complete specimens collected).

The majority of the females that were regenerating contained oocytes in the coelom cavity (about 68%).

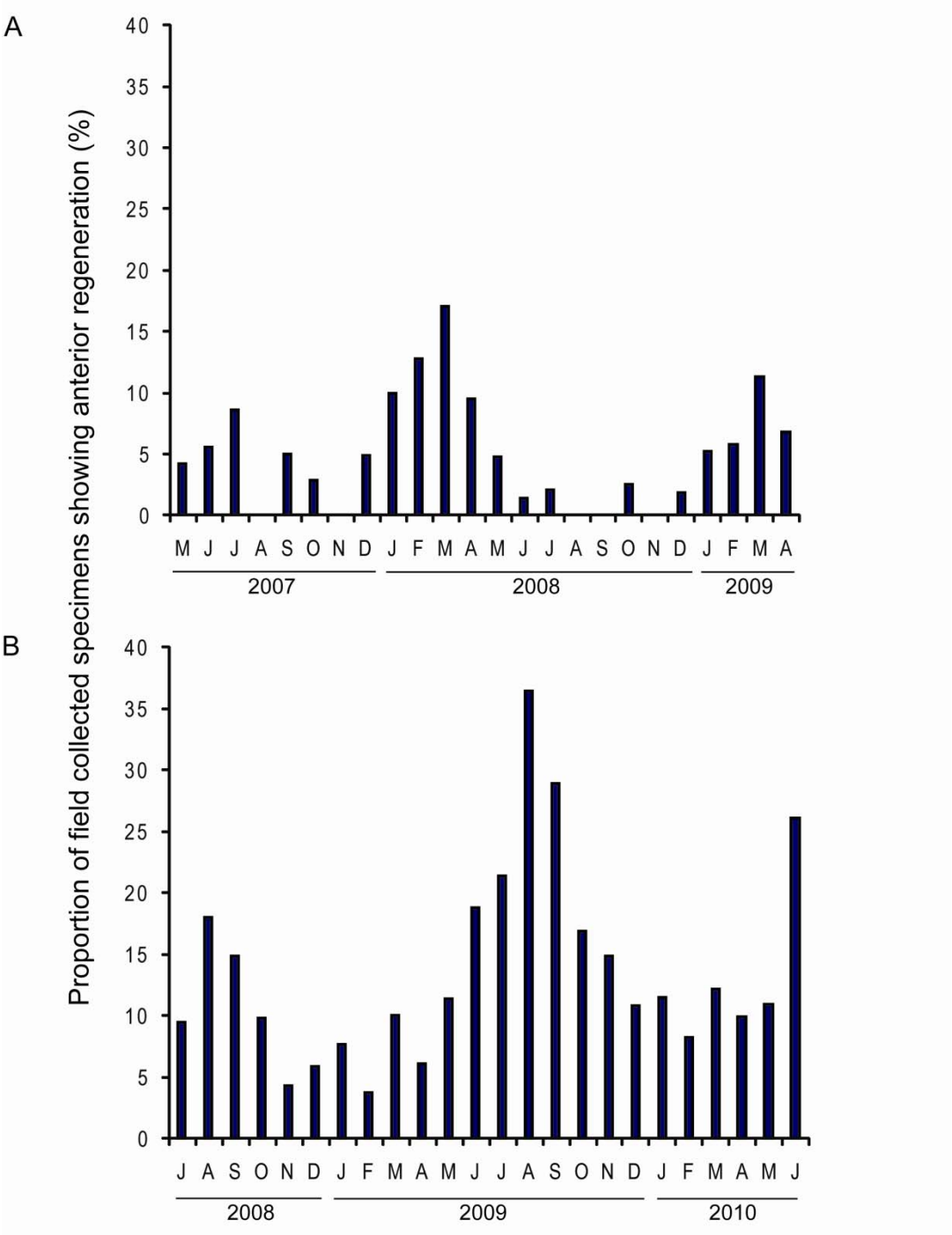

Figure 28 – Proportion of field collected *Diopatra* specimens regenerating anterior end (A) – *D. neapolitana*; (B) – *D. marocensis*.

4.4 Discussion

The capacity of various *Diopatra* species to regenerate anterior and posterior portions as been previously reported (Paxton and Bailey-Brock, 1986; Bely, 2006; Safarik et al., 2006; Budaeva and Fauchald, 2008; Berke et al., 2009; Pires et al., 2010; Pires et al., 2011; Table 10). This capacity as been reported mainly from field collected specimens, so it usually refers to a small number of specimens and gives no data on the regeneration time gap nor the surviving proportion of specimens (cf. Table 10). Berke et al. (2009) studied in the laboratory the ability of *D. cuprea* to regenerate the anterior end. These authors stated that 7 days after regeneration they had specimens with a small regenerated anterior end and at day 14 they had a single specimen fully regenerated, while the regenerated chaetigers width was between 30% to 80% of the older chaetigers (Berke et al., 2009).

According to the present study, *D. neapolitana* specimens regenerating the anterior end need from 4 to 6 days to heal the amputated region, from 10 to 12 days to have the regenerated anterior end differentiated and start feeding, and from 50 to 70 days to reach full regeneration, i.e., when the regenerated portion presents the same width as the rest of the body. When the posterior end is being regenerated, then from 4 to 10 days were needed to heal the amputated region, from 12 to 18 days to have a differentiated regenerated portion and from 50 to 70 days were necessary for a complete regeneration of the chaetigers.

Budaeva and Fauchald (2008) described five regeneration stages for *D. tuberculantennata*, based on observations by Scanning Electronic Microscopy of 12 field collected specimens regenerating the anterior ends. Those stages were not possible to discriminate in the present study, as the individuals were only observed under a stereomicroscope. Nevertheless, Budaeva and Fauchald (2008) first development stage includes individuals with a small globular prostomium surrounded by 5 protuberances that will be the antennae and palps, and at stage II they already observe the prostomium, peristomial ring and chaetigers, perceptible as ventro-lateral protuberances starting to differentiate ventrally. The second stage described by Budaeva and Fauchald (2008) should correspond to the protuberance reported in the present study at day 6 to 8, when the prostomial appendages were still not observed.

Table 10 - Summary of regenerative characteristics known for the genus *Diopatra*, and others Onuphidae species (1 - % refers to the proportion of the sampled population).

Species	Nr. specimens regenerating (1)	Portion regenerating	Nr. maximun chaetigers regenerating	Observations	Reference
D. aciculata	More than 30%	Posterior	-	Regenerating posterior ends frequently at high densities, revealing aggressive encounters among polychaetes	Safarik et al., 2006
D. sugokai as *D. amboinensis*	-	Anterior	-	-	Pflugfelder, 1929
D. cuprea	27	Antennae, anterior and posterior	Anterior: 11.6 ± 1.4; posterior: 0 to 75	-	Berke et al., 2009
D. dexiognatha, as *D. leuckarti*	Anterior: 11.8%; Posterior: 7.2%	Posterior and anterior	-	-	Bayley-Brock, 1984
D. marocensis	Anterior: 234 (13.6%); Posterior 5 (0.29%)	Anterior and posterior	Anterior: 4 to 11	The majority of regenerating specimens, about 68%, had oocytes in coelom cavity	Present study
D. micrura	Some anterior, 1 Posterior	Anterior and posterior	-	-	Pires et al., 2010
D. neapolitana	Anterior: 77 (5%); Posterior: 6 (0.3%)	Anterior and posterior	Anterior: 2 to 13; posterior 25 to 90	The majority did not contain gametes, except some females, with small oocytes	Pires et al., 2011, present study
D. tuberculantennata	12	Anterior	more than 9 - 10	-	Budaeva and Fauchald, 2008
D. dentata	2	Posterior		-	Paxton, 1993
D. maculata	2	Posterior	-	1 with attached larvae in the tube	Paxton, 1993
Americonuphis magna	0	Antennae and posterior	-	Were not able to regenerate anterior ends, only the antennae	Berke et al, 2009
Onuphis striata	1	Posterior	30	30 chaetigers regenerating without branchiae	Fauchald, 1982
Nothria mannarensis	1	Posterior	-	Posterior end narrower than rest of the body	Fauchald, 1982
Onuphis pectinata	1	Anterior	-	Anterior end narrower than the rest of the body	Fauchald, 1982

Stages III and IV, for which ceratophores had rings, the prostomium, peristomium and chaetigers were distinctly differentiated and the peristomial cirri were absent, are comparable to our observations at days 10 to 12 after amputation. At stage V, the individuals showed very well differentiated chaetigers, all the prostomial structures as well as spiraled branchiae with up to 4 filaments and the regenerated portion was about 50% the width of the normal chaetigers. This stage is comparable to our observations at day 20. Budaeva and Fauchald (2008) stated that these 5 regeneration developmental stages are similar to the ontogenesis in genus *Diopatra*. In fact, the development of the prostomial structures on larvae is firstly characterized by the appearance of 5 small buds, which will be the future antennae and palps (Pires et al., 2011). Peristomial cirri appear late, as well as the branchiae (Conti and Massa, 1998). A similar development pattern was observed in the present study, as well as by Budaeva and Fauchald (2008). The main noticed difference between the ontogenesis and the regeneration stages, also reported by Budaeva and Fauchald (2008), was the development of the chaetigers, that in the larvae occurs first, and in the regeneration development occurs only after the prostomium and peristomium development.

Field regenerating specimens in this study were regenerating in mean 9.0 ± 2.51 chaetigers (4 to 13), and subacicular hooks started from chaetiger 17. In the laboratory, *D. neapolitana* specimens regenerated 3 to 13 anterior chaetigers, depending on the amputation level. When specimens were amputated at begin of the branchiae (after chaetigers 3 or 4), they regenerated the same number of chaetigers. Specimens amputated at chaetiger 10 regenerated 8 to 10 chaetigers, and specimens amputated at chaetiger 15 regenerated 9 to 13 chaetigers. These results showed that usually fewer chaetigers are regenerated than those lost. *D. marocensis* regenerating specimens regenerated also less chaetigers than lost. Uninjured specimens presented subacicular hooks from chaetiger 13 to 15, and in regenerating specimens they were found from chaetigers 12 to 15.

Comparing the percentage of field regenerating individuals of *D. marocensis* and *D. neapolitana*, it seems that the first species is more affected by predator's attacks than *D. neapolitana* in Ria de Aveiro. This could be due to the smaller size

of *D. marocensis*, to the fact that the species is slower escaping to the interior of the tube than *D. neapolitana*, or even to the reproduction mode which tends to produce a more aggregate distribution of individuals when compared to *D. neapolitana*.

Safarik et al. (2006), observed *D. aciculata* specimens regenerating posterior chaetigers. These authors stated that the number of individuals regenerating posterior chaetigers increased with increasing worm density, suggesting that posterior losses (possibly autonomy) was caused by aggressive encounters between polychaetes. These aggressive encounters were more frequent at higher densities, caused by the competition for space (Safarik et al., 2006). A similar behavior was previously reported by Bridges et al. (1996), with the polychaete *Neanthes arenaceodentata*.

In this study, we show that *D. neapolitana* has the ability to regenerate either an anterior or a posterior end, if the specimens are not amputated close to their mid branchial region. In that case, neither the anterior nor the posterior portions survive. When cut up to chaetiger 15, the posterior end regenerates the anterior part of the body and when cut beyond chaetiger 25, the anterior end regenerate the posterior part of the body. This study also shows that the regeneration success expressed as percent survival is higher when fewer branchial chaetigers are lost. These results indicate that *D. neapolitana* should survive predator attacks if these remove a few anterior chaetigers. What concerns bait digging, as usually more than 20 chaetigers are harvested by collectors (Cunha et al., 2005), our results indicate that the posterior part that remains inside the tube will not be able to regenerate an anterior end. This contradicts the common believe from bait diggers which mention that their activity will not affect the species due to the “seed” they leave in the sediment when harvesting. This should be alluding to the ability of the posterior end to regenerate a new specimen, but this capacity is jeopardize, as indicated in this study, when 20 or more anterior chaetigers are collected. Without such regenerative capacity, this implies that the activity of bait diggers must be managed in order to avoid overexploitation of the resource. Such overexploitation in Ria de Aveiro could actually be taking place, as according to Freitas et al. (2011), in a study conducted in Mira channel, bait diggers collected about 2.9

ind.m^{-2} in 2001/2002 but only 1.6 ind.m^{-2} were harvested in 2007/2008. These authors suggest that the reason behind the decrease of bait collection in the Mira Channel was the reduction in numbers of bait diggers on this channel, who preferred to move to other channels located in the Northern part of Ria de Aveiro. Such displacement of the bait diggers could be related to a stock decrease, not being profitable to continue exploiting the Mira channel. Dagli et al. (2005) also mentioned that in Izmyr Bay, Turkey, each digger required about 10h of effort to collect 2000 specimens, but ten years before the same could be harvested in just one hour, indicating the stock was being overexploitated (Dagli et al*.,* 2005). The present study advises to take management actions in order to sustain the exploitation of natural *D. neapolitana* populations.

Chapter 5

Final remarks

5.1 Concluding remarks

At the beginning of this study *D. neapolitana* was the only species from this genus recognized for Europe. This work showed that two other *Diopatra* species could be found in Portugal, *D. marocensis*, described for the Moroccan coast, and *D. micrura* sp. nov., a new species to the science. In the meanwhile, *Diopatra* sp. (not yet described) was found in Arcachon Bay (France) by Berke *et al.* (2010). The present study compared genetically and morphologically, using characters that have not been used previously, these four European *Diopatra* species.

In Ria de Aveiro, *D. neapolitana* occurs sympatrically with *D. marocensis* and *D. micrura* being *D. neapolitana* the most abundant and widely distributed. *D. marocensis* was observed only in four localities and *D. micrura* in two, both more abundant near the entrance of the Ria. *D. micrura* inhabits the lower infralitoral and *D. marocensis* and *D. neapolitana* are distributed along the medio and infralitoral areas. These two last species display very different reproductive patterns: *D. neapolitana* is a broadcast spawning, with free-swimming larvae, and *D. marocensis* broods in the parental tube with direct development. *D. micrura* and *Diopatra sp* from Arcachon seem to be also free spawners, as oocyte diameter ranged between 140 and 200 µm for *D. micrura*, and 120 to 220 µm for *Diopatra sp.*

For *D. neapolitana* it was observed individuals with gametes inside the coelom all year round, but the peak reproductive period occurred between May and August, when almost all individuals had gametes in the coelom and females contained more oocytes than at any other time of the year.

All year round it was observed eggs and larvae inside the female tubes of *D. marocensis*, with peak values from April to October, and the reproduction peak from March to September. In the same month, it was found tubes of *D. marocensis* with larvae in different developmental stages, as well as tubes with eggs, suggesting that this species is asynchronous, as at the same time it was observed different development stages, indicating that the species may spawn at any time of the year.

The male: female sex ratio along the year is very different in *D. neapolitana* and *D. marocensis*, being about 1:1 in *D. neapolitana* and from 1:2 to 1:4 in *D. marocensis*. Apart from a colour difference in mature specimens due to gametes in the coelom, no other morphological difference between males and females were found for both species.

The presence of the three species in some places of Ria de Aveiro is an excellent opportunity to follow the installation of *D. marocensis* and *D. micrura* in the Ria, and to study their interaction with *D. neapolitana*, especially the interaction between *D. neapolitana* and *D. marocensis*, as they present different reproductive patterns.

Diopatra species were observed regenerating posterior and anterior segments, as also as some structures from the prostomium. With this work we concluded that *D. neapolitana* is able to regenerate if no more than 20th anterior chaetigers are removed and thus should survive predator attacks but this species doesn't recover when cut by fishermen. These results reinforce the need to manage the activity of bait diggers in order to avoid overexploitation of the resource. Such overexploitation in Ria de Aveiro could actually be taking place, as according to Freitas et al. (2011), in a study conducted in Mira channel, bait diggers collected about 2.9 ind.m^{-2} in 2001/2002 but only 1.6 ind.m^{-2} were harvested in 2007/2008. These authors suggest that the reason behind the decrease of bait collection in the Mira Channel was the reduction in numbers of bait diggers on this channel, who preferred to move to other channels located in the Northern part of Ria de Aveiro. Such displacement of the bait diggers could be related to a stock decrease, not being profitable to continue exploiting the Mira channel. Dagli et al. (2005) also mentioned that in Izmyr Bay, Turkey, each digger required about 10h of effort to collect 2000 specimens, but ten years before the same could be harvested in just one hour, indicating the stock was being overexploitated (Dagli et al*.,* 2005).

Although *D. neapolitana* to be intensively exploited as live fish bait in Ria de Aveiro, no management or conservation regulations are currently set for the species and the existing legislation is very scarce. In the case of the Sado estuary,

located about 350 Km south of Ria de Aveiro, harvesting of *D. neapolitana*, *Marphysa sanguinea* and *Hediste diversicolor* is not allowed from November 1st until April 30th (Portuguese legislation: Portaria nº 576/2006). That period is reported in the legislation as coincident with spawning and juvenile growth. However, this is not supported by the present or other studies. The main reproductive period for *H. diversicolor* in the Sado estuary was from April to August/September (unpublished data). In the Southwestern coast of Portugal (Odeceixe, Aljezur, and Carrapateira) the same species was reported as reproducing during all year, with important peaks in September and May (Fidalgo e Costa, 2003). In Ria de Aveiro, the species also showed two important reproductive periods, in March and September (Abrantes et al., 1999). The reproductive period of *Marphysa sanguine*a was mainly from March/April to October/November in the Sado estuary (unpublished data) and a peak spawning period in April-May was reported from the Venice Lagoon (Italy) by Prevedelli et al. (2007). For *Diopatra neapolitana,* the present study and Dagli et al. (2005), in Izmir Bay (eastern Mediterranean), show that the main reproduction peak was from May to August.

In Portugal, with the exception of the resting period established for the Sado estuary, the exploitation of polychaetes occurs all year, being more intense in warm months. Cancela da Fonseca and Fidalgo e Costa (2008) observed that the capture of these species has increased in recent years and that the mean size of harvested individuals is smaller.

The digging activity has negative impacts on the whole ecosystem. The benthic community is affected as a whole and so are the species which depend on it for food (mainly birds and fishes). In addition, the biogeochemical cycles could be affected and the release of nutrients and bio-availability of metals enhanced (Cancela da Fonseca and Fidalgo e Costa, 2008). All of this emphasizes the urgent need for a sustainable exploitation of these natural resources, not only in Ria de Aveiro but in all coastal areas. The use of scientifically supported legislation coupled with control in the allocation of bait-digging licenses and with regular monitoring of the impacted areas should be implemented. Mitigation measures could be applied either by restricting the harvest (in the case of *D. neapolitana* in

Ria de Aveiro, the most suitable period seems to be April until September) or by establishing yearly-rotating resting-areas. The rotation system has been suggested as an effective solution to minimize the negative impacts of this kind of resource exploitation by Fowler (1999), Cancela da Fonseca and Fidalgo e Costa (2008), and is being used in Korea, which is one of the largest exporters of polychaetes in the world (Choi, 1985).

Chapter 6

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ANNEXES

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Diopatra neapolitana and *Diopatra marocensis* from the Portuguese coast: Morphological and genetic comparison

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ABSTRACT

This paper reports the presence of *Diopatra marocensis* in European waters, for which *Diopatra neapolitana* was the only species recognized until recently. Both species coexist in transitional waters, where *D. marocensis* may be mistaken for young specimens of *D. neapolitana*. The population of *D. marocensis* studied in the coastal shelf can be traced back to 1997 and is increasing in density, apparently benefiting from a local anthropogenic organic enrichment source.

This study emphasizes the main morphological characteristics that allow discriminating the two species and uses a molecular approach through the mitochondrial DNA genes 16S rDNA and COI (cytochrome c oxidase subunit I) analysis to confirm their distinction. The percentage of nucleotides divergence of the 16S and COI genes between the two species was 14% and 17%, respectively. The nucleotide sequence was conserved among all specimens of the same species for 16S gene, and the differences observed between individuals of the same species for the COI gene always corresponded to a silent alteration with no amino acid change. The nucleotide sequences of the two genes of both species were also compared to the sequences of *Diopatra aciculata* deposited in the EMBL database. The divergence values between *Diopatra marocensis* and *D. aciculata* were 14% and 18% for 16S and COI, respectively whereas between *Diopatra neapolitana* and *D. aciculata* were 1% and 5% for 16S and COI, respectively. Phylogenetic analysis was performed to deduce relationships among the *Diopatra* species studied. This analysis showed that *D. marocensis* and *D. neapolitana* are in different clades and thus could be considered different species, whereas *D. aciculata* and *D. neapolitana* are in sister clades thus emphasising their similarities, already noticed at a morphological level.

1. Introduction

The genus *Diopatra* Audouin and Milne-Edwards, 1833 comprises common onuphid polychaetes living in intertidal and shallow subtidal areas of all major oceans (Paxton, 1986). Species of this genus can reach high densities in many habitats (Cunha et al., 2005; Dagli et al., 2005) and play an important ecological role by stabilizing the sediment with their tubes, increasing its structural complexity and potentially enhancing the sediment biodiversity (Bailey-Brock, 1984) while facilitating the settlement and attachment of some algal species (Thomsen and McGlathery, 2005).

The genus *Diopatra* is characterized by the presence of tentacular cirri and spirally arranged branchial filaments. It is best represented in warmer waters and comprises about 50 species worldwide (Budaeva and Fauchald, 2008). In Australia, this genus is represented by seven species (Paxton, 1993), whereas in Europe only *Diopatra neapolitana* Delle Chiaje, 1841 has been recognized until recently. *Diopatra neapolitana* has been reported in intertidal and shallow subtidal habitats, namely in the Red Sea and Indian Ocean (Wehe and Fiege, 2002), the Mediterranean Sea (Gambi and Giangrande, 1986; Arvanitides, 2000; Dagli et al., 2005), the Pacific Ocean (Choe, 1960) and the Atlantic Ocean (Fauvel, 1923; Moreira et al., 2006; Lourido et al., 2008). Wethey and Woodin (2008) set the northern limit of *D. neapolitana* in France, in Pointe de Penvins (Brittany), and later, in Berke et al. (in press) set it only to the French–Spanish border, about 460 km to the south of the previous point, and consider that the species found in northern areas is new to science. Other authors had previously expressed their uncertainty about the cosmopolitan distribution of *D. neapolitana*. Day (1967) noted that several closely related species to *D. neapolitana* had been misidentified and that all records of the species outside the Mediterranean Sea could be considered doubtful. Paxton (1993) also noted that specimens reported by Choe (1960)

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as *D. neapolitana* could possibly belong to the species *Diopatra sugokai*. On the contrary, *Diopatra aciculata* from Australia is very similar to *D. neapolitana* and Paxton (1993) stated that although she could not observe distinct differences between the two species, retained *D. aciculata* as a separate taxa until more information to the contrary would become available. *Diopatra* taxonomy at the specific level seems still not clear and the need for a major revision has been recognized (Paxton, 1986), which is in fact a mandatory step prior to the study of species distributional range shifts.

Diopatra marocensis has been recently described by Paxton et al. (1995), from individuals collected off the Moroccan Atlantic coast. The species was recorded outside its type locality by Pires et al. (2008), in a number of sites along the Portuguese coast and also by Berke et al. (in press), who mention the species for the lagoon of Óbidos, western coast of Portugal. The present study confirms the widespread distribution of *D. marocensis* in Portuguese waters, with records on the western and the southern coast. It also gives temporal data obtained in a shelf area in western Portugal subject to anthropogenic organic enrichment (Silva et al., 2004), which traces the presence of the species in European waters as far as 1997. This study also emphasizes the main morphological characteristics that allow the distinction between *D. marocensis* and *Diopatra neapolitana* while using a molecular approach to confirm the distinction between the two species by characterising two mitochondrial DNA genes, 16S rDNA (16S ribosomal RNA gene) and COI (cytochrome c oxidase subunit I) (Halanych and Janosik, 2006). Finally, based on the data obtained in this study and records from the gene bank for 16S rDNA and COI, it compares *Diopatra aciculata* (Knox and Cameron, 1971) to *D. neapolitana* and *D. marocensis*.

2. Methods

2.1. Sampling

Specimens of *Diopatra marocensis* and *Diopatra neapolitana* were collected along the western Portuguese coast, in Ria de Aveiro (A), the Lagoon of Óbidos (B), and Guia, a shelf area located off the Tagus estuary (C) and along the southern coast in the near shelf off Olhão (D) (Fig. 1).

In Ria de Aveiro, a few intertidal sites were specifically chosen to study the *Diopatra* populations. Here the sediment was collected with a shovel up to 30 cm deep and the *Diopatra* tubes were gently removed from the sediment and taken to the laboratory for live observations and examination of the specimens. In the other localities the biological material from previous sampling programmes was re-examined for taxonomic confirmations. In these ecosystems, the sites studied were all subtidal and the sediment samples were taken using a Smith–McIntyre grab (0.1 m^2, Guia) or a Ponar grab (0.05 m^2, Lagoon of Óbidos and the Southern Coast). Sediment was washed through a 1 mm mesh sieve and the material retained was fixed with 4% formalin neutralized with borax.

From the Guia and Ria de Aveiro systems, some *Diopatra marocensis* individuals were also collected for genetic studies. The Guia specimens were preserved in ethanol (96%) and those from Ria de Aveiro were kept cold during field sampling and frozen at the laboratory (−20 °C). For the same purpose, individuals of *Diopatra neapolitana* were sampled in Ria de Aveiro and handled as *D. marocensis* specimens collected in this system.

In order to describe the sedimentary environment, a sediment sample from each site was collected. Samples for grain size analysis were stored in plastic containers and for the total volatile solids (TVS) analysis samples were stored in a cold environment and frozen at −20 °C in the laboratory.

2.2. Laboratory procedures

In the laboratory, 602 specimens of *Diopatra marocensis* (78 from Ria de Aveiro, 50 from the Lagoon of Óbidos, 474 from Guia) and 243 specimens of *Diopatra neapolitana* from Ria de Aveiro were examined for the morphological comparison. The individuals were identified and measured, for total length and the width of chaetiger 10 (without parapodia). The numbers of chaetigers, rings in the ceratophores, whorls of the branchia and teeth in the pectinate chaeta were recorded. The last chaetiger with branchiae was registered. The colour pattern and the form of the prostomium of the two species were described, based upon the observation of live specimens. The density data presented in this study for *D. marocensis* includes juvenile and adult specimens. Distinction between adults, juvenile and larvae was made using the proposed description in Fadlaoui et al. (1995).

A more detailed morphological study was based on scanning electron microscopy (SEM). Specimens stored in 70% ethanol were dehydrated in graded ethanol series and critical point dried in a Bal-Tec CPD-030 critical point dryer, using ethanol as a transition fluid. After drying, specimens were sputter coated with gold: palladium alloy 40:60 in a Polaron sputter coating system. SEM micrographs were taken in a JEOL JSM-5400 scanning microscope. The photos presented in this work were based on two specimens for *Diopatra marocensis* and one for *Diopatra neapolitana*.

Sediment grain size was analysed by wet and dry sieving following Quintino et al. (1989) and sediment was characterized regarding the grain size classes: gravel (particles with diameter above 2 mm), sand (0.063–2.000 mm) and fines content (<0.063 mm). The amount of sediment in each grain size class was expressed as a percentage of the whole sediment, dry weight. The data were used to calculate the median value, P_{50}, expressed in phi (ϕ = -$\log_2$mm) units, corresponding to the diameter that has half the grains (dry weight) finer and half coarser. Given that no detailed grain size analysis was performed for the fines fraction, the median could not be calculated for the samples with more than 50% fines content. These sediment samples were classified as mud. Sands were classified using the median, expressed in ϕ units, according to the Wentworth scale (Doeglas, 1968): very fine sand (median between 3 and 4 ϕ); fine sand (2–3 ϕ); medium sand (1–2 ϕ) or coarse sand (0–1 ϕ). The final classification adopted the description "clean", "silty" or "very silty", when fines were ranging from 0% to 5%, from 5% to 25%, and from 25% to 50%, respectively, of the total sediment, dry weight (Quintino et al., 1989).

Total volatile solids were determined as weight loss on ignition at 450 °C during 5 h (Byers et al., 1978; Kristensen and Anderson, 1987) of 1 g sediment sample after an initial drying at 60 °C for 24 h.

2.3. DNA extraction

Specimens of *Diopatra marocensis* collected in the Guia area (15 specimens) and Ria de Aveiro (30 specimens), and specimens of *D. neapolitana* collected in Ria de Aveiro (45 specimens), were used for the genetic analyses. Total genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions. Purified DNA was aliquoted in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C, until required.

2.4. PCR amplification of 16S/COI genes

Partial regions of the mitochondrial 16S rDNA (~500 bp) and cytochrome c oxidase subunit I (COI) (~700 bp) genes were amplified by PCR using the following primers: 16S rDNA: 16SarL (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SbrH (5′-

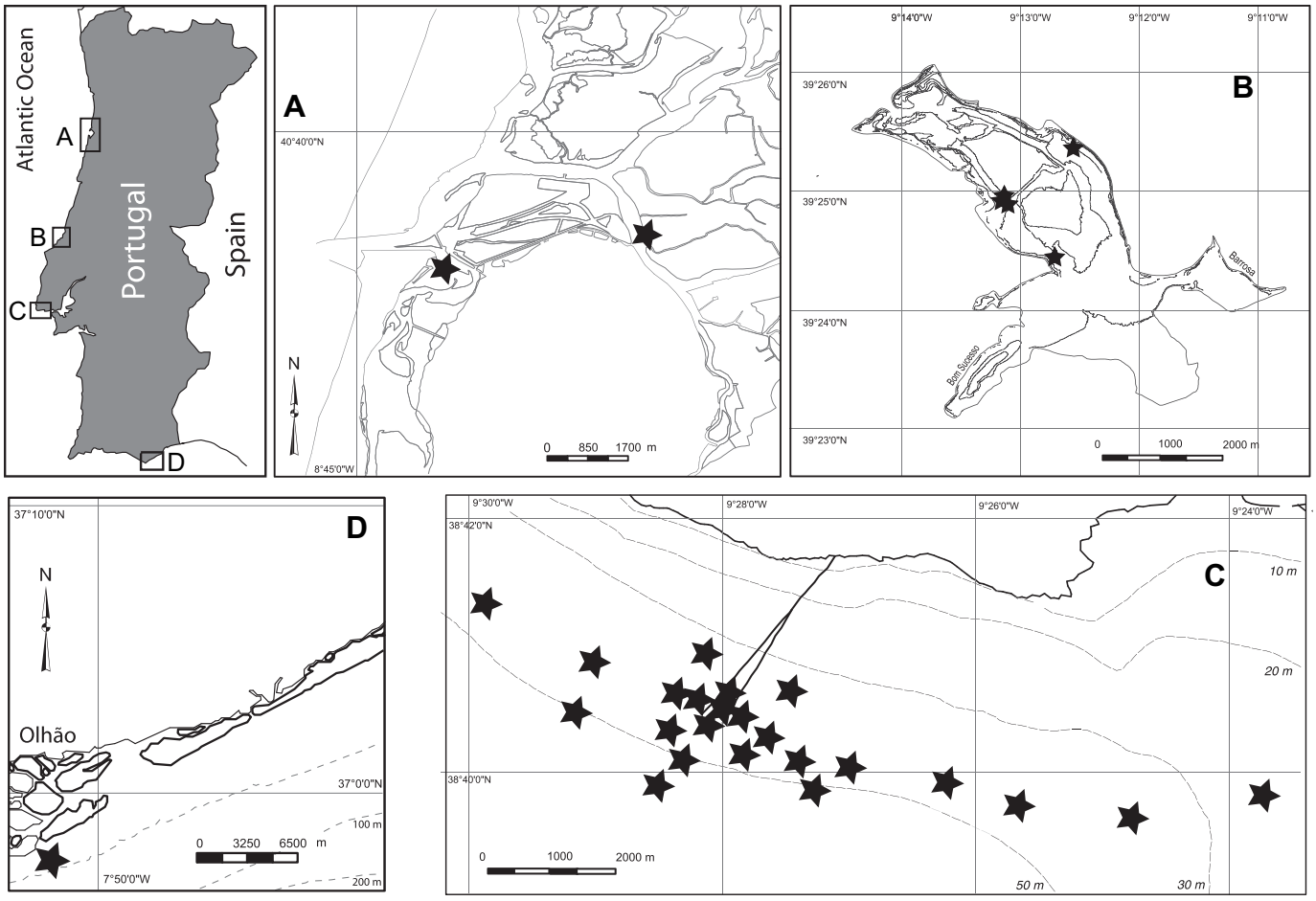

Fig. 1. Sampling areas where *Diopatra marocensis* was found. A – Ria de Aveiro; B – Lagoon of Óbidos; C – Guia, coastal shelf off Tagus estuary; D – shelf off Olhão. ★ – presence.

CCGGTCTGAACTCACATCACGT-3′) (Palumbi et al., 1991); COI: LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994).

PCR reactions were performed in a final volume of 50 µl containing 10–100 ng of genomic DNA, 1 µM of each primer, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega) and 0.5 U Taq DNA polymerase (Promega). Amplification occurred on a MJ Mini Thermal-Cycler (Citomed) with the following thermal cycling parameters: initial denaturation at 94 °C, 3 min, followed by 34 cycles of: denaturation at 94 °C, 1 min; primer-specific annealing 49 °C (16S rDNA) or 45 °C (COI), 30 s; extension at 72 °C, 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 sizes of the amplicons.

2.5. DNA sequencing and analysis

Nucleotide sequencing of each PCR-amplified fragment (16S/COI) on both orientations and from two independent reactions were commercially performed (STAB Vida, Portugal).

Sequences were analysed using the Biological Sequence Alignment Editor BioEdit version 7.0.0 (free software by Tom Hall, Department of Microbiolgy, North Carolina State University). The obtained sequences were compared to others deposited in the EMBL GenBank nucleotide sequence databases. Genetyx-WIN Version 5.1 (Software Development, Tokyo) was employed to determine the percentage of homology between *Diopatra marocensis* and *Diopatra neapolitana*, for both genes and also to compare with others sequences deposited in the EMBL database for the species *Diopatra aciculata*. Additional COI and 16S sequences of Eunicida species were obtained from GenBank to complement the analysis of the species being studied. These sequences were analysed together in a single data set, with *Ophryotrocha alborana* as an outgroup. The data set sequences were aligned in MEGA 3.1 (Kumar et al., 2004) with CLUSTALW using the default alignment settings.

The phylogenetic analyses were conducted with the computer program MEGA version 3.1 (Kumar et al., 2004) by applying Neighbor Joining (NJ). To verify the robustness of the internal nodes of NJ trees, bootstrap analysis was carried out using 1000 pseudo replicates.

3. Results

3.1. Morphological comparison between Diopatra marocensis and Diopatra neapolitana

The terminology used in this work to describe the morphology of both species followed Paxton (1998) and is presented in Fig. 2.

The main features to discriminate *Diopatra neapolitana* from *Diopatra marocensis* are summarized in Table 1 which also includes the characteristics referred to by Paxton et al. (1995) for *D. marocensis* and Fauvel (1923) and Dagli et al. (2005) for *D. neapolitana*.

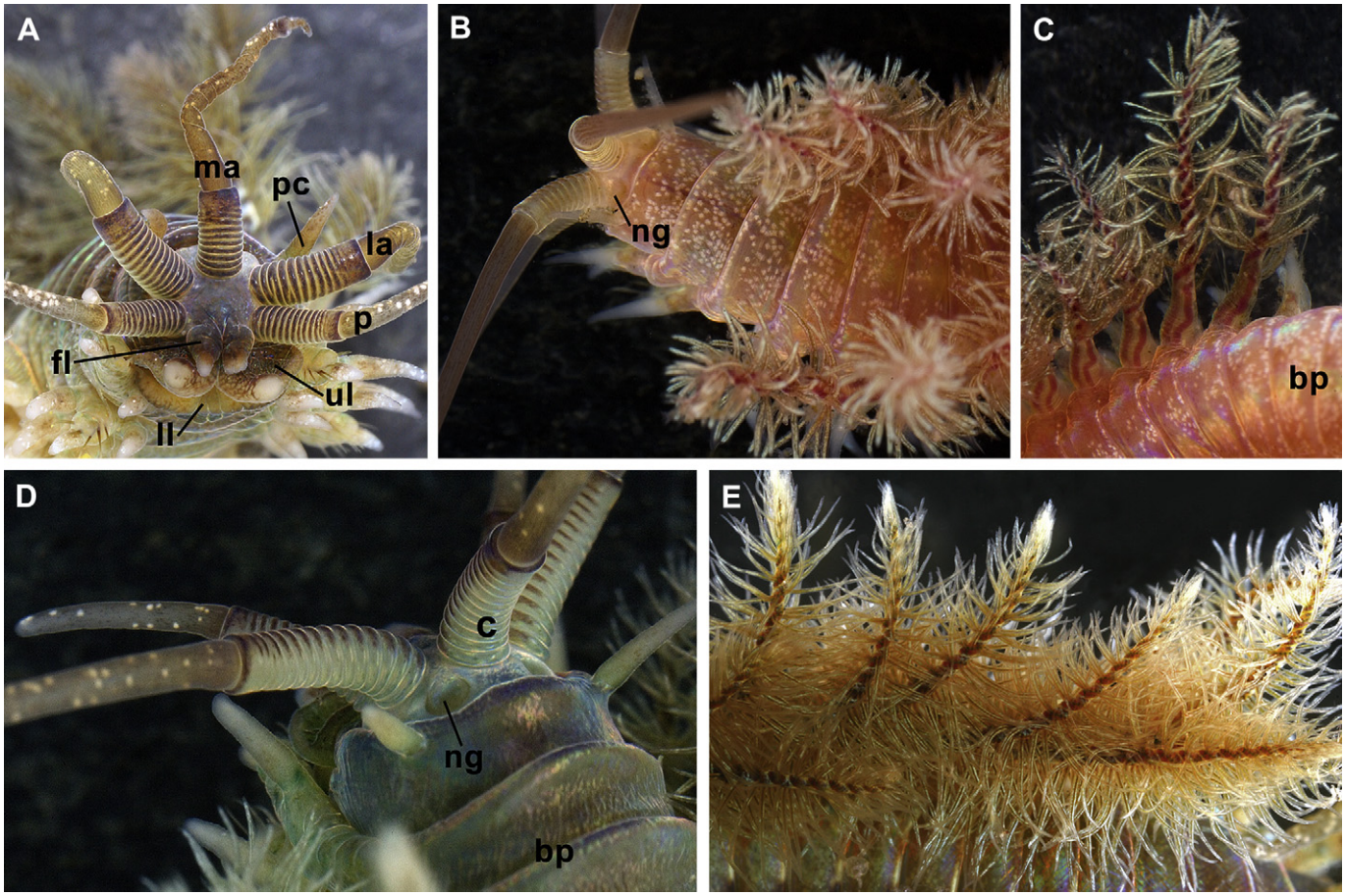

Fig. 2. Morphological characteristics of live specimens of *Diopatra marocensis and D. neapolitana*: A – prostomial and peristomial appendages of *D. neapolitana* (sensu Paxton, 1998); B – anterior end of *D. marocensis*, dorsal view; C – branchiae of *D. marocensis*; D – anterior end of *D. neapolitana*, dorsal view; E – branchiae of *D. neapolitana*. bp – brown patch; c – ceratophores; fl – frontal lip; la – lateral antenna; ll – lower lip, ma – median antenna; ng – nuchal groove; p – palp; pc – peristomial cirrus; ul – upper lip.

In their adult stage, the two species present different sizes, with *Diopatra marocensis* being smaller than *Diopatra neapolitana* both in terms of body length and width and also with fewer numbers of chaetigers (cf. Table 1).

The colour pattern, observed in live individuals, varied in both species but a general pattern can be described: *Diopatra marocensis* presented a pinkish colour (Fig. 2B) with more whitish parapodia. The prostomium and the ceratophores showed a brown pigmentation, with the area of the nuchal grooves more whitish. The frontal lips were also whitish but brown pigmented at the base. Along the segments, both species presented a brown mid-dorsal patch, in our specimens (Fig. 2C and D) forming a line along the medium dorsal anterior part of the body, up to chaetigers 15–20 in *D. marocensis*, (width: 0.5–1 mm), and chaetiger 30–40 in *Diopatra neapolitana* (width: 1–1.4 mm). Almost all individuals presented many white irregular small spots on the anterior end, dorsal view (Fig. 2B). In *D. neapolitana* such white spots are more evident on the antennae and palps (Fig. 2D). However the white spots are not visible in preserved specimens. The overall colour in *D. neapolitana* is iridescent greenish (Fig. 2D). In the males, the body area with gametes acquires a cream colour in the reproductive period. The frontal lips of *D. neapolitana* were brown from the base up to the middle of their length (Fig. 2A). Brown rings in the ceratophores were clear on the antennae and palps (Fig. 2A and D). The nuchal

Table 1
Morphological characteristics used to distinguish *D. neapolitana* from *D. marocensis*. The values given for the present study correspond to the mean ± standard deviation, with the range between brackets ($n = 35$ for each species).

	Present study		Paxton et al. (1995)	Fauvel (1923)	Dagli et al. (2005)
	D. marocensis	*D. neapolitana*	*D. marocensis*	*D. neapolitana*	
Length (cm)	8.93 ± 1.98	36.39 ± 13.50	3.5	15–50	34.7
Width of 10th chaetiger (mm)	2.97 ± 0.66	7.08 ± 1.68	2.0–4.5	–	7.74
Number of chaetigers	141.69 ± 22.80	269.20 ± 31.16	112	200–300	239
Colour	Pinkish	Greenish iridescent	Pale	Greenish iridescent	Brownish
Number of rings of the ceratophores	8.54 ± 0.82 (6–9)	15.40 ± 0.50 (15–16)	7–9	–	15–16
Nuchal grooves	Crescentic	Rounded	Crescentic	–	Sub-triangular
Chaetiger where branchiae begin	4.14 ± 0.36 (4–5)	4.46 ± 0.51 (4–5)	4–5	4–5	–
Maximum number of branchial whorls	7.80 ± 1.13 (6–9)	16.20 ± 1.18 (14–18)	6–9	–	14
Chaetiger where branchiae finish	33.77 ± 2.96 (26–38)	64.40 ± 3.53 (56–70)	30–41	60–70	65
Limbate serration of the chaetae	On shelf	All border line	On shelf	–	Coarsely serrated
Nr of teeth of the pectinate chaetae	14.69 ± 2.01 (11–20)	6.60 ± 1.33 (5–10)	11–20	6–9	6–10

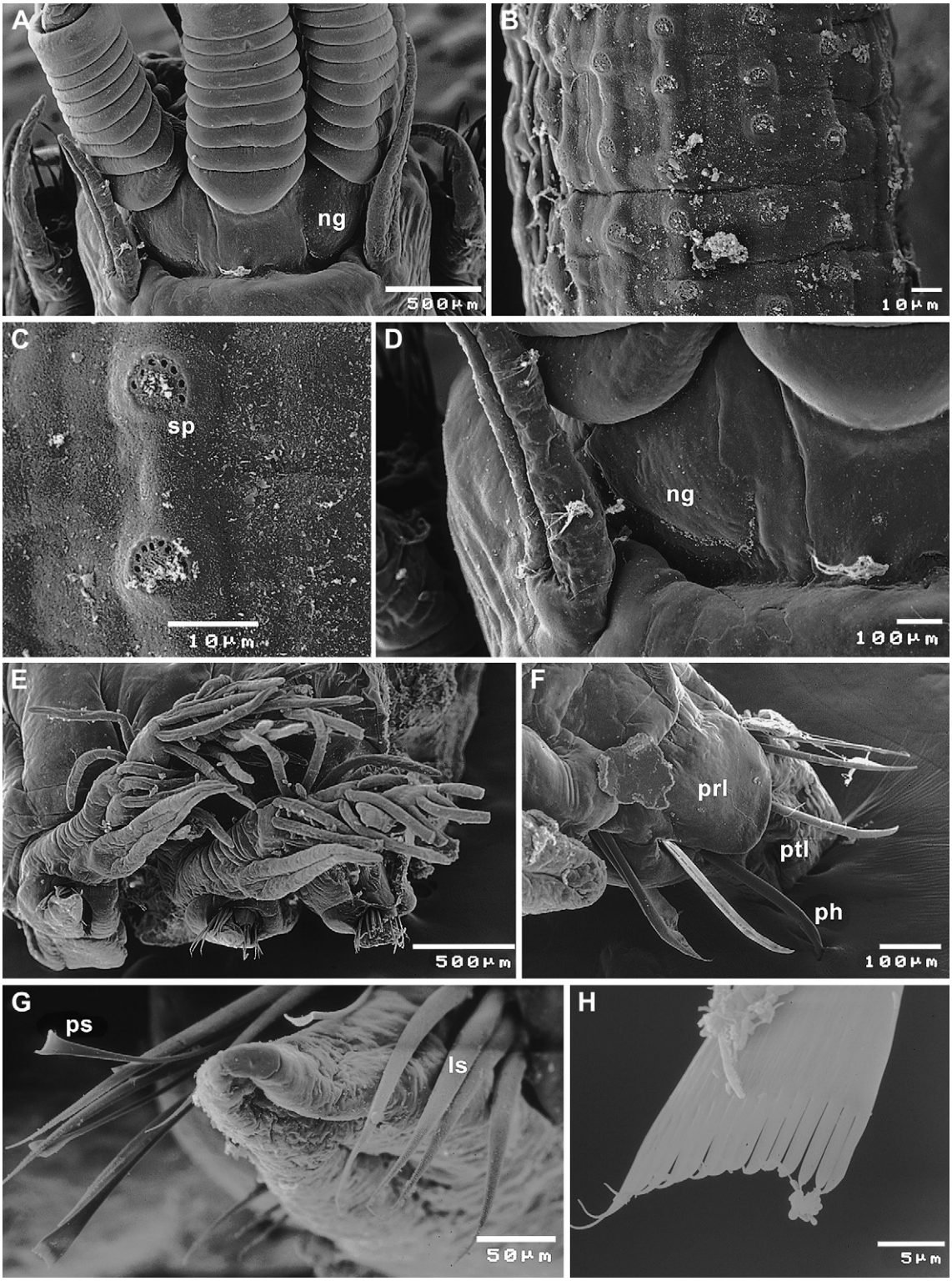

Fig. 3. Scanning electron micrographs of *Diopatra marocensis*: A – prostomium, dorsal view; B – rows of sensory papillae on antenna; C – enlarged sensory papillae of antenna; D – nuchal groove; E – branchiae; F – modified parapodium; G – parapodium of chaetiger 6; H – chaetiger pectinate chaeta. ls – limbate chaeta; ng – nuchal groove; ph – pseudo-compound hook; prl – prechaetal lobe; ps – pectinate chaeta; ptl – postchaetal lobe; sp – sensory papilla.

grooves were greenish and lighter than the prostomium. Up to the first six chaetigers the overall colour is of a dark brown, becoming lighter and greenish in the rest of the body.

The prostomium was rounded anteriorly in *Diopatra marocensis* and slightly pointed in *Diopatra neapolitana*. The ceratophores of *D. marocensis* antennae and palps presented 6–8 proximal rings and 14–15 rings in *D. neapolitana,* with a longer distal ring in both species (Figs. 2B and D; 3A; 4A; cf. Table 1). The antennae and palps, with interrupted longitudinal rows of sensory papillae, 16–18 in the case of *D. marocensis* (Fig. 3B and C) and 20–22 for *D. neapolitana*

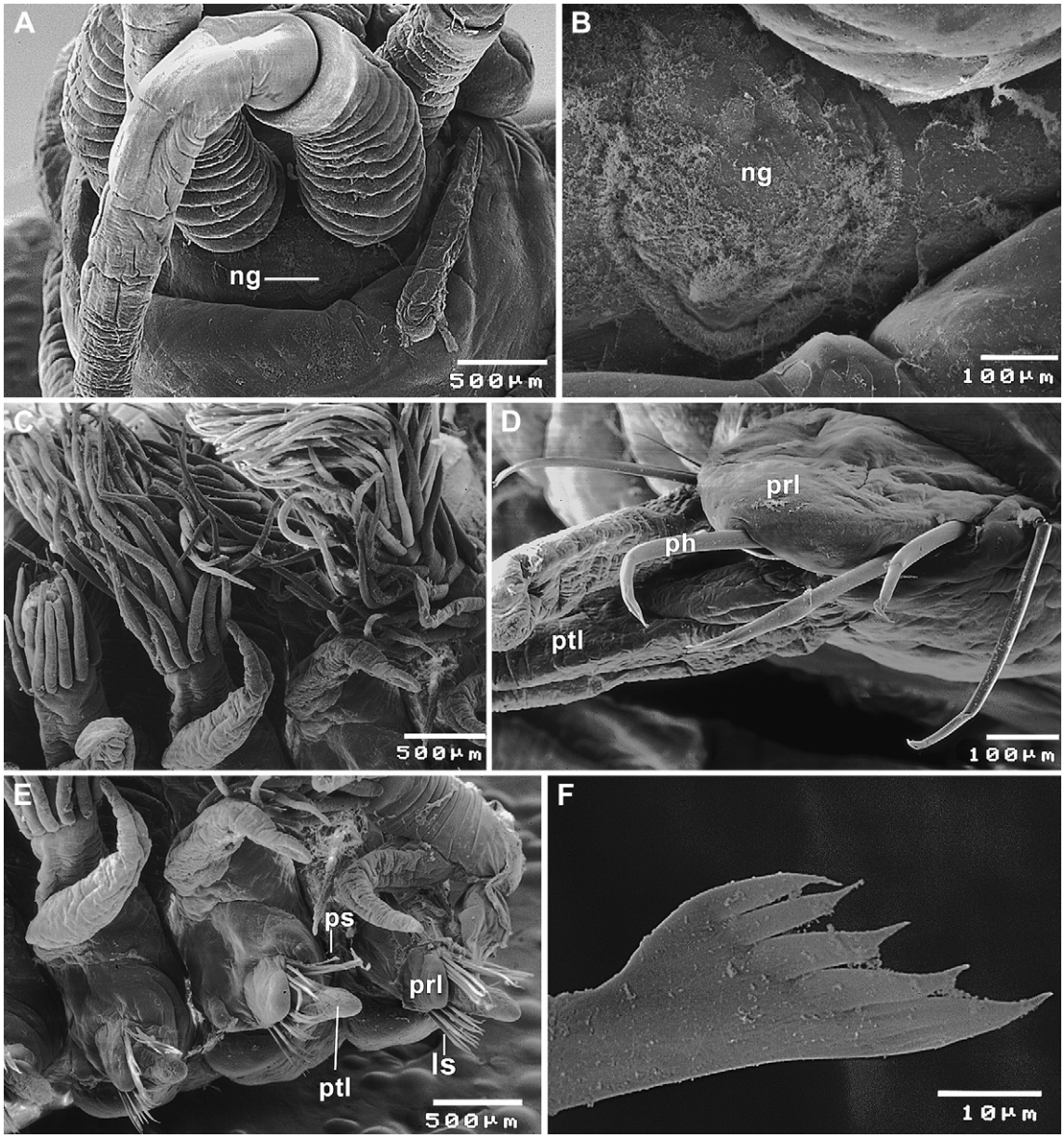

Fig. 4. Scanning electron micrographs of *Diopatra neapolitana*: A – prostomium, dorsal view; B – nuchal groove; C – branchiae; D – modified parapodium; E – parapodium of chaetigers 5, 6 and 7; F – pectinate chaeta. ls – limbate chaeta; ng – nuchal groove; ph – pseudocompound hook; prl – prechaetal lobe; ps – pectinate chaeta; ptl – postchaetal lobe.

have been observed at SEM. The nuchal grooves were crescentic in *D. marocensis* and rounded in *D. neapolitana* (Figs. 2B and D; 3A and 3D; 4A and B) and the peristomial cirri were about twice the length of the peristomium in both species.

Four larger modified parapodia (in the first four chaetigers), with rounded prechaetal and subulate postchaetal lobes were observed in both species (Figs. 3F; 4D). The prechaetal lobes were observed up to chaetigers 6–10 in *Diopatra marocensis* and up to 15–20 in *Diopatra neapolitana* (Fig. 4E). Ventral lobes are absent in *D. marocensis*. In *D. neapolitana* they are present from chaetiger 5 to about 20–25. They are most distinct on setigers 10–20, then shifting more dorsally, forming the new presetal lip by chaetiger 20–25. Spiralled branchiae appeared from chaetigers 4 or 5 in both species. They were best developed from chaetigers 5 to 9 with 6–9 whorls in *D. marocensis* and from chaetigers 7 to 9 with 14–18 whorls in *D. neapolitana* (Figs. 2C and E; 3E; 4C; cf. Table 1). The branchial filaments became gradually slender towards posterior chaetigers and were absent from chaetigers 30–41 in *D. marocensis* and from 60–70 in *D. neapolitana* (cf. Table 1).

Concerning the chaetae, *Diopatra marocensis* presented bidentate and *Diopatra neapolitana* uni- to bidentate pseudocompound hooks (Figs. 3F and 4D) with pointed hoods and two rows of blunt small spines along their shafts and limbate chaetae, on the first four modified parapodia. In the remaining parapodia limbate and pectinate chaetae appeared together with bidentate subacicular hooks from chaetigers 13–15 in *D. marocensis* and 19–25 in *D. neapolitana*. Limbate and pectinate chaetae have different morphology in the two species. Limbate chaetae in *D. neapolitana* were coarsely serrated along almost the whole borderline while in *D. marocensis* they were only serrated on the shelf. Pectinate chaetae have 11–20 teeth in *D. marocensis* with slender base up to the tip (Fig. 3H) while *D. neapolitana* presented 5–10 wider teeth, slenderer in the tip than in the base (Fig. 4F; cf. Table 1).

The pygidium was similar in both species with two pairs of anal cirri.

3.2. Genetic analysis

In *Diopatra marocensis,* all individuals analysed displayed identical nucleotide sequences for the 16S gene. For the COI gene two haplotypes were found. Two individuals from Guia presented a base alteration at position 404, where a nucleotide adenine was replaced by a cytosine (TCA to TCC), corresponding to a silent alteration with no amino acid change. All specimens of *D. marocensis* from Ria de Aveiro had the same nucleotide sequence.

For *Diopatra neapolitana*, two haplotypes were also observed in COI gene. A base alteration occurred at position 560, where a nucleotide cytosine was replaced by a thymine (CTC to CTT), corresponding to a silent substitution with no amino acid change. In this case, 24 individuals had a CTC codon and 21 the CTT. For the 16S gene, all individuals of *D. neapolitana* shared the same nucleotide sequence.

The percentage of nucleotide divergence (nucleotide substitution) of the 16S and COI genes (mean percentage in the case of COI), between *Diopatra marocensis* and *Diopatra neapolitana,* was 14% and 17%, respectively. For COI, deduced amino acid sequence comparison between both species revealed that they differ in 4 amino acids, showing 1.74% of divergence. The majority of the differences in nucleotides between both species occurred at the third position of the codon and therefore corresponds to silent alterations.

The nucleotide sequence of 16S and COI genes of both species were compared with the nucleotide sequence of *Diopatra aciculata* deposited in the EMBL database (Struck et al., 2006; COI: AY838867, 16S: AY838826). The mean divergence values (nucleotide substitution), between *Diopatra marocensis* and *D. aciculata,* were 18% and 14% for COI and 16S, respectively. These values were similar to the ones obtained in this study when comparing *D. marocensis* and *Diopatra neapolitana*. However, the percentage of divergence between *D. neapolitana* and *D. aciculata* was of 5% and 1% for COI and 16S, respectively.

Phylogenetic analysis from both genes (Fig. 5) clearly shows that *Diopatra neapolitana* and *Diopatra marocensis* are in two different clades, however *D. neapolitana* and *D. aciculata* are very close, in a sister clade.

COI and 16S nucleotide sequences from *Diopatra marocensis* and *Diopatra neapolitana* were deposited at EMBL database, under the accession numbers: *D. marocensis* 16S – J473306, COI – FJ646632 and GQ456165 and *D. neapolitana* 16S – EU878538, COI – EU878539 and GQ456164.

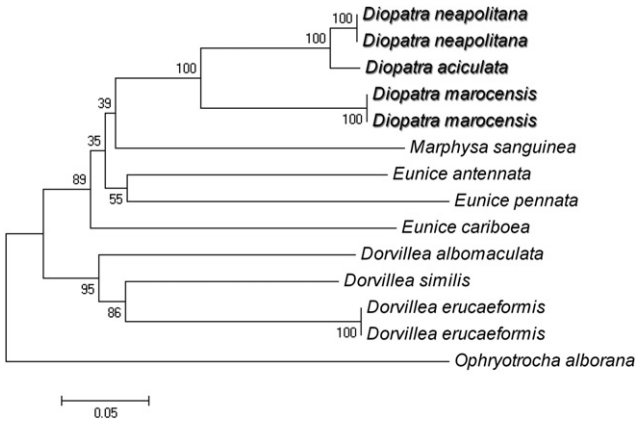

Fig. 5. Phylogenetic analysis of the data set containing the 16S and COI sequences of Eunicida species. Numbers near the nodes indicate the percent bootstrap values. The branch length indicator represents 0.05 substitutions per site.

Table 2
Sediment characteristics from the sampling areas where *Diopatra marocensis* was found (TVS – Total Volatile Solids).

Locality	Sediment type	Fines content (%)	TVS (%)
Ria de Aveiro	Very silty fine sand	34.4	4.5
	Silty fine sand	24.7	4.3
Lagoon of Óbidos	Mud	71.3	5.0
	Very silty very fine sand	48.5	6.5
	Silty fine sand	15.8	1.9
	Clean medium sand	0.8–2.1	0.6
Guia	Clean fine sand	3.5–4.91	1.1–1.46
	Silty fine sand	5.1–12.4	1.2–1.6
	Silty very fine sand	16.5	2.2
Olhão	Clean fine sand	1.0	–

3.3. Diopatra marocensis distribution along the Portuguese coast

At the present *Diopatra marocensis* has been identified along the western and southern Portuguese coast (Fig. 1): in 23 of the 30 sites sampled in the Guia area, in the vicinity of a submarine outfall (April 1997–October 2007), in 5 of the 107 sites sampled in the Lagoon of Óbidos (July 2002), in 2 sites of Ria de Aveiro of a survey covering all the Ria (October 2008) and in 1 of the 92 sites of a survey covering Olhão to Vila Real de St° António. The sediment types, percentage of fines and percentage of total volatile solids (TVS) of the sites where *D. marocensis* was found are presented in Table 2. The species has been found in a range of sediments (mud, very fine sand, fine sand and medium sand), with fines ranging from 71.3% to 0.8% and total volatile solids from 6.5% to 1.1%.

Fig. 6 presents the temporal evolution of the *Diopatra marocensis* abundance in the Guia area, where annual sampling has been regular since 1994. This species was first noticed in April 1997, in one site (0.66/0.1 m^2, juveniles) and then in October 1998 (0.66/0.1 m^2, adults), also in a single site. It was only after October 1999 that the species began to show a clear distribution pattern, centred on the submarine outfall (cf. Fig. 6). Since October 2002, besides adults and juveniles an increasing number of larvae have been noticed: 10 in 2002; 208 in 2003; 153 in 2004; 32 in 2006 and 123 larvae in October 2007 (numbers correspond to larvae/0.1 m^2 and are not included in the abundance data presented in Fig. 6).

4. Discussion

The present study presents a morphological and genetic comparison between the species *Diopatra neapolitana* and *Diopatra marocensis*, recording the presence of *D. marocensis* on European coasts. This species was first mentioned outside the Moroccan coast by some authors of the present paper, in the annual meeting of the Portuguese Ecological Society (Pires et al., 2008) and latter, in the lagoon of Óbidos, by Berke et al. (in press), being its distribution along the Southern Portuguese coast referred for the first time in the present paper.

The available temporal data allow tracing back to 1997 the presence of *Diopatra marocensis* on the western coast of Portugal, where the species was initially misidentified as *Diopatra neapolitana.* In fact, after reanalysis of the collected material, no specimens of *D. neapolitana* were registered in this shelf area off the Tagus estuary. In Ria de Aveiro, the present study is the first concerning *Diopatra* species and there is no material from older surveys to analyze. As such, it is not possible to ascertain if the presence of *D. marocensis* in this system is only recent. However a general survey of the intertidal areas of the whole system, in October 2008, showed that at present only two sites near the entrance of the Ria are colonized by *D. marocensis* whereas *D. neapolitana* presents

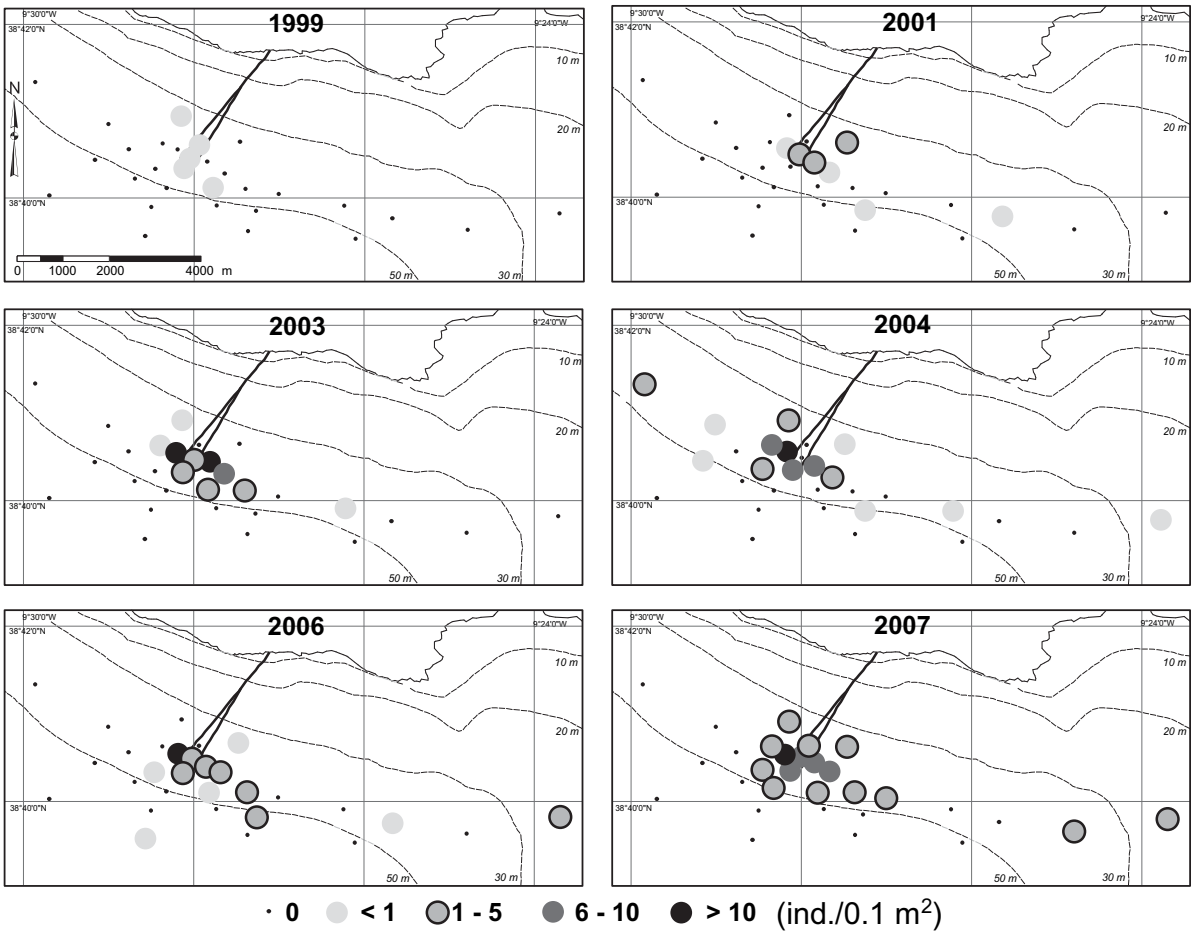

Fig. 6. Density of *Diopatra marocensis* in the Guia shelf area. Samples in all years were taken in early Autumn (October).

a much wider distribution. The species could then be new to this system. If this is the case, this is particularly interesting because of the possible competition between *D. marocensis* and *D. neapolitana,* knowing that the latter represents an important economic natural resource for the local community (Cunha et al., 2005). This also suggests that *D. marocensis* could be spreading to the North, eventually due to climate change. In fact, the wide distribution of this species along the Portuguese coast, the proximity of the Moroccan coast and also the way this natural resource is commercially exploited in Portugal is not in favour of an accidental introduction by bait trade, as suggested by Berke et al. (in press). Bait trade of *Diopatra* in Portugal is mainly for a local market or otherwise only for exportation. The exploited populations are all natural and at the present there are no cultivated areas based upon imported specimens.

In the Guia shelf area, off the Tagus estuary, *Diopatra marocensis* is spreading its distribution, and is increasing in density, centered in areas around the sewage outfall. This suggests that the species is benefiting from the anthropogenic organic enrichment. In fact, previous studies have shown that the superficial sediments grain size in this area did not change since the pre-operational phase of the outfall, whereas a community shift was detected with the installation of opportunist species (Quintino et al., 2001; Silva et al., 2004). This was also consistent with the detection in the same areas, of organic carbon and nitrogen from terrestrial origin in sediments and faunal tissue (Sampaio et al., in press). The reproduction strategy of *D. marocensis*, producing larvae which develop inside the adult tube, without a pelagic phase (Fadlaoui et al., 1995), allows the species to immediately benefit from the additional organic matter source, and facilitate its local spread. Although this population in the shelf area off the Tagus estuary is installed mainly over clean fine sand, *D. marocensis* was found in sediments ranging from mud to clean medium sand, a wider variety than that known for *Diopatra neapolitana* which is common in muds and muddy sands (Fauvel, 1923; Bellan, 1964; Gambi et al., 1998).

Diopatra neapolitana and *Diopatra marocensis* are clearly two different species showing morphological distinctions such as size, nuchal grooves, number of the rings in the ceratophore, chaetae (Fauvel, 1923; Paxton et al., 1995; Dagli et al., 2005) and colour pattern, corroborated by genetic evidence concerning the mitochondrial DNA genes, 16S and COI genes. In *D. marocensis and D. neapolitana* all individuals analysed displayed identical nucleotide sequence for the 16S gene while for the COI gene two haplotypes were found. For the COI gene, deduced amino acid sequences of both species differ in four amino acids, which correspond to a 1.74% divergence. However, they are replaced by others of the same chemical group (Stryer, 1999) and therefore the sequences are translated in proteins of the same family that will have the same function.

COI and 16S rDNA are considered conserved genes in the mitochondrial genome, but a nucleotide divergence of respectively, 17% and 14% is frequent in polychaetes. For example, for 16S rDNA, in the syllid genus *Autolytus,* the mean percentage of nucleotide divergence, based on 16 species, is 21%, with a range of 28–17% (Nygren and Sundberg, 2003), for the genus *Eunice* it is 14% (based on 3 species sequences deposit in the GenBank; range: 13–17%) and for the genus *Lumbrinereis* is 13% (based on 4 species sequences; range:

12–14%). With respect to the COI gene in the genus *Dorvillea* the nucleotide mean sequence divergence is 22% (based on 3 species sequences; range: 20–23%) and in the genus *Lumbrinereis* is 20% (based on 4 species sequences; range: 18–22%). These comparisons suggest that the genetic variation between *Diopatra neapolitana* and *Diopatra marocensis* is within a normal range for polychaetes. But the genetic comparison between *D. neapolitana* and *D. aciculata* is below, 5% and 1% for COI and 16S rDNA, respectively, emphasizing the similarity between these two species.

Phylogenetic relationships among Eunicida species show that *Diopatra marocensis* and *Diopatra neapolitana* are in different clades and may be considered different species. However, *Diopatra aciculata* and *D. neapolitana* are in a sister clade. Dagli et al. (2005) and Paxton (1993) stated that *D. aciculata* is very similar to *D. neapolitana* concerning its morphological appearance and chaetae types. The results presented in this work emphasize the similarities between these two species at a genetic level. Despite the fact that some differences occur they can be expected between distant populations of a species (note that the EMBL database information for *D. aciculata* concerns an Australian population). However being the COI and 16S conservative genes in the mtDNA genome, better in showing differences than similarities, further studies using for instance, faster mtDNA markers or quicker nuclear markers, like microsatellites, are desired, to lead to a reliable conclusion about the taxonomic validity of *D. aciculata*.

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Diopatra (Annelida: Onuphidae) diversity in European waters with the description of *Diopatra micrura*, new species

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Abstract

This study describes a new species of the genus *Diopatra* Audouin and Milne-Edwards, 1833. *Diopatra micrura* **sp. nov.**, was found on the western and the southern coast of Portugal and can be distinguished from other *Diopatra* species by a characteristic striped colour pattern of the antennae and palps. Other diagnostic morphological characteristics include ventral parapodial lobes, crescentic nuchal organs, ceratophores with 12–15 rings, and subacicular hooks from chaetigers 8–13. This species was found mainly in fine or very fine sand with variable fines content, from the intertidal region up to 50 meters depth.

Molecular studies of mitochondrial DNA genes 16S rDNA and COI confirmed the distinction of *D. micrura* **sp. nov.**, from other European *Diopatra* species. The percentage of nucleotides divergence between the new species and *D. neapolitana* and *D. marocensis* was respectively 16% and 17% for COI and 12% and 15% for 16S. The nucleotide sequence for the 16S gene was always the same in all specimens of *D. micrura* and two haplotypes were found for the COI gene. The discovery of *D. micrura* **sp. nov.**, brings the number of *Diopatra* species known from Portugal to three and from Europe to four; a key to the four species is provided.

Key words: Taxonomy, striped antennae, 16S rDNA, COI, distribution, Portugal

Introduction

The genus *Diopatra* Audouin and Milne-Edwards, 1833 includes about 50 species distributed around the world (Budaeva & Fauchald 2008). These onuphid polychaetes are common in intertidal and shallow subtidal areas of all major oceans although better represented in warmer waters (Paxton 1986). The genus is characterised by the presence of peristomial cirri and spiralled branchiae (Paxton 1986).

Diopatra neapolitana Delle Chiaje, 1841 was until very recently the only recognised species of *Diopatra* in European waters. Recent studies revealed the presence of *Diopatra marocensis* Paxton *et al.*, 1995 in Portugal (Rodrigues *et al.* 2009) and a species reported as *Diopatra* sp. A from Arcachon to Dunquerque, France, by Berke *et al.* (2010).

The present paper reports the discovery of another species, *Diopatra micrura*, **sp. nov.**, increasing to three the number of *Diopatra* species known from Portugal and to four the number of European species.

Besides the morphological description, this study also uses a molecular approach to confirm the distinction of *D. micrura* **sp. nov.** from *D. neapolitana*, *D. marocensis*, and *Diopatra* sp. from Arcachon Bay, by characterising two mitochondrial DNA genes, 16S rDNA (16S ribosomal RNA gene) and COI (cytochrome c oxidase subunit I) (Halanych & Janosik 2006). It also presents the distribution of *D. micrura* **sp. nov.**, along the Portuguese coast together with the sediment type and depth of occurrence.

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Material and methods

Sampling. On the western coast of Portugal, specimens of *D. micrura* were collected in Ria de Aveiro, near the mouth and in intertidal areas, on the adjacent shelf area, on the shelf off Nazaré and in Guia, off the Tagus Estuary; on the southern coast, specimens were collected near the Guadiana river mouth (Fig. 1, Table 1).

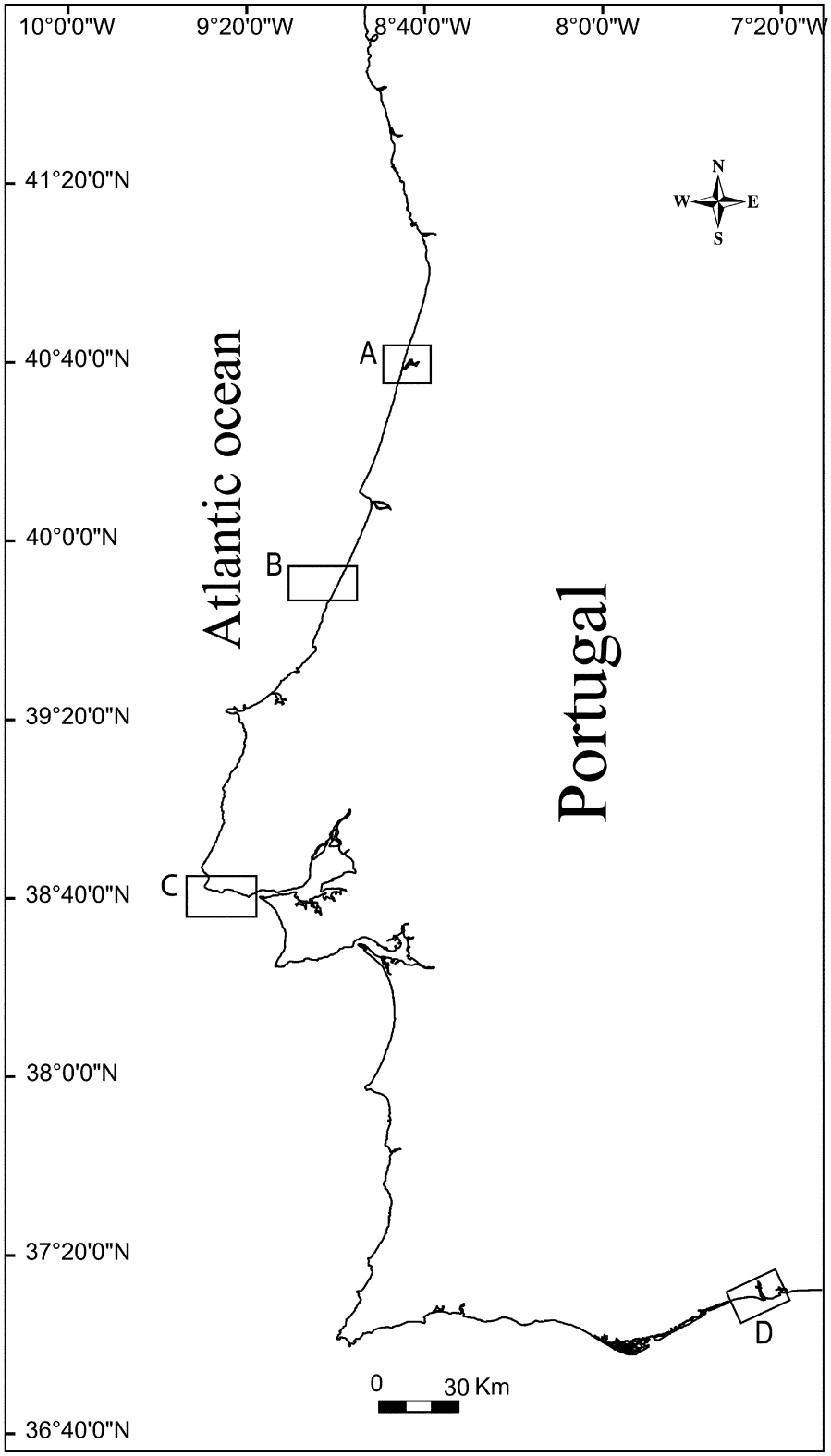

FIGURE 1. Sampling areas where *Diopatra micrura* **sp. nov.**, was found: A—Aveiro (shelf and Ria); B— coastal shelf off Nazaré; C—Guia, coastal shelf off Tagus estuary; D—coastal shelf off Guadiana estuary

TABLE 1. List of sites where *Diopatra micrura* **sp. nov.** was sampled. AS—Shelf off Aveiro; NS—Nazaré; RA—Ria de Aveiro; GS—Shelf off Guadiana River; TS—Shelf off Tagus Estuary; SVT - Total Volatile Solids (organic matter).

Site	Latitude (ºN)	Longitude (ºW)	Depth (m)	Date	Sediment type	Fines content (%)	SVT (%)
TS1	38° 39' 45.840"	9° 25' 40.440"	40	Mar-94	Silty fine sand	9.03	2.71
TS1A	38° 39' 45.840"	9° 25' 40.440"	40	Apr-01	Silty fine sand	9.03	2.71
TS1B	38° 39' 45.840"	9° 25' 40.440"	40	Oct-03	Silty fine sand	9.03	2.71
TS2	38° 40' 29.340"	9° 27' 59.580"	40	Jan-97	Clean fine sand	3.4	1.43
TS3	38° 40' 33.600"	9° 28' 11.640"	40	Jan-97	Clean fine sand	4.47	1.41
TS3A	38° 40' 33.600"	9° 28' 11.640"	40	Oct-01	Clean fine sand	4.47	1.41
TS3B	38° 40' 33.600"	9° 28' 11.640"	40	Oct-03	Clean fine sand	4.47	1.41
TS4	38° 40' 20.820"	9° 28' 7.440"	45	Jan-97	Clean fine sand	3.52	1.12
TS4A	38° 40' 20.820"	9° 28' 7.440"	45	Oct-01	Clean fine sand	3.52	1.12
TS4B	38° 40' 20.820"	9° 28' 7.440"	45	Oct-03	Clean fine sand	3.52	1.12
TS5	38° 40' 25.080"	9° 27' 50.640"	40	Jan-97	Clean fine sand	3.36	1.39
TS5A	38° 40' 25.080"	9° 27' 50.640"	40	Apr-97	Clean fine sand	3.36	1.39
TS6	38° 40' 37.620"	9° 28' 22.080"	40	Jan-97	Clean fine sand	5.32	1.49
TS6A	38° 40' 37.620"	9° 28' 22.080"	40	Oct-98	Clean fine sand	5.32	1.49
TS6B	38° 40' 37.620"	9° 28' 22.080"	40	Apr-01	Clean fine sand	5.32	1.49
TS7	38° 40' 51.060"	9° 29' 0.180"	40	Jan-97	Silty medium sand	10.06	1.41
TS7A	38° 40' 51.060"	9° 29' 0.180"	40	Oct-97	Silty medium sand	10.06	1.41
TS8	38° 41' 20.220"	9° 29' 51.600"	40	Jan-97	Clean coarse sand	0.81	1.28
TS8A	38° 41' 20.220"	9° 29' 51.600"	40	Oct-02	Clean coarse sand	0.81	1.28
TS9	38° 40' 1.560"	9° 29' 41.640"	34	Jan-97	Clean fine sand	1.57	1.4
TS10	38° 40' 37.620"	9° 27' 53.940"	38	Apr-97	Clean fine sand	2.07	1.30
TS10A	38° 40' 37.620"	9° 27' 53.940"	38	Oct-02	Clean fine sand	2.07	1.30
TS11	38° 40' 20.100"	9° 28' 27.120"	45	Apr-97	Silty fine sand	5.08	1.45
TS12	38° 40' 16.920"	9° 27' 38.460"	40	Apr-97	Clean fine sand	3.71	1.34
TS12A	38° 40' 16.920"	9° 27' 38.460"	40	Oct-01	Clean fine sand	3.71	1.34
TS13	38° 40' 38.820"	9° 27' 27.540"	26	Apr-97	Clean fine sand	3.88	1.61
TS14	38° 40' 2.640"	9° 27' 0.480"	40	Apr-97	Silty fine sand	5.20	1.50
TS14A	38° 40' 2.640"	9° 27' 0.480"	40	Oct-99	Silty fine sand	5.20	1.50
TS14B	38° 40' 2.640"	9° 27' 0.480"	40	Oct-03	Silty fine sand	5.20	1.50
TS14C	38° 40' 2.640"	9° 27' 0.480"	40	Jan-06	Silty fine sand	5.20	1.50
TS15	38° 40' 56.100"	9° 28' 8.400"	26	Oct-99	Clean fine sand	3.87	1.28
TS15A	38° 40' 56.100"	9° 28' 8.400"	26	Oct-02	Clean fine sand	3.87	1.28
TS16	38° 40' 51.060"	9° 29' 0.180"	40	Apr-01	Silty medium sand	10.06	1.41
TS17	38° 39' 53.640"	9° 28' 29.940"	60	Oct-01	Silty very fine sand	14.29	2.21
TS18	38° 39' 51.060"	9° 27' 16.500"	50	Oct-02	Silty very fine sand	24.87	3.39
TS19	38° 40' 15.109"	9° 26' 30.275"	35	Oct-02	Clean fine sand	2.83	1.2
TS20	38° 40' 4.881"	9° 24' 9.258"	27	Oct-02	Clean fine sand	4.50	1.6
TS21	38° 39' 49.183"	9° 23' 43.095"	26	Oct-02	Silty very fine sand	11.12	1.6
TS22	38° 40' 6.180"	9° 27' 23.880"	40	Oct-03	Clean fine sand	4.10	1.84
TS23	38° 39' 49.200"	9° 23' 43.080"	26	Oct-03	Silty fine sand	11.05	2.52
TS23A	38° 39' 49.200"	9° 23' 43.080"	26	Oct-04	Silty fine sand	11.05	2.52
TS23B	38° 39' 49.200"	9° 23' 43.080"	26	Oct-07	Silty fine sand	11.05	2.52

to be continued.

TABLE 1. (continued.)

Site	Latitude (ºN)	Longitude (ºW)	Depth (m)	Date	Sediment type	Fines content (%)	SVT (%)
TS23C	38° 39' 49.200"	9° 23' 43.080"	26	Oct-08	Silty fine sand	11.05	2.52
TS23D	38° 39' 49.200"	9° 23' 43.080"	26	Sep-09	Silty fine sand	11.05	2.52
TS24	38° 39' 54.600"	9° 26' 14.340"	40	Oct-04	Silty fine sand	5.33	1.73
TS24A	38° 39' 54.600"	9° 26' 14.340"	40	Jan-06	Silty fine sand	5.33	1.73
TS25	38° 40' 7.440"	9° 27' 48.420"	45	Jan-06	Clean fine sand	4.56	1.54
TS26	38° 40' 33.720"	9° 28' 45.900"	45	Oct-07	Silty fine sand	5.82	1.60
AS1	40° 40' 50.637"	8° 46' 45.085"	15	Dec-02	Clean very fine sand	1.6	0.7
AS2	40° 41' 11.718"	8° 46' 32.926"	15	Dec-02	Clean very fine sand	1.61	0.84
AS3	40° 41' 4.855"	8° 47' 8.918"	18	Dec-02	Clean very fine sand	1.88	0.97
AS4	40° 40' 38.579"	8° 46' 26.623"	13	Dec-02	Clean very fine sand	1.67	0.92
AS5	40° 41' 0.196"	8° 46' 15.844"	13	Dec-02	Clean very fine sand	1.34	0.72
AS6	40° 41' 56.762"	8° 46' 6.409"	15	Dec-02	Clean very fine sand	1.18	0.71
GS1	37° 10' 21.119"	7° 28' 2.219"	12	May-07	Silty fine sand	5.03	-
GS2	37° 8' 52.440"	7° 24' 59.281"	10	May-07	Very silty very fine sand	46.14	-
NS	39° 50' 43.901"	9° 6' 40.799"	37.2	Apr-08	Silty fine sand	6.65	0.94
RA1	40° 38' 28.896"	8° 44' 0.276"	Intertidal	Oct-08	Silty fine sand	24.7	4.3
RA2	40° 38' 28.896"	8° 44' 0.276"	Intertidal	Mar-09	Silty fine sand	24.7	4.3
RA3	40° 38' 28.896"	8° 44' 0.276"	Intertidal	Sep-09	Silty fine sand	24.7	4.3
RA4	40° 38' 28.896"	8° 44' 0.276"	Intertidal	Nov-09	Silty fine sand	24.7	4.3

In Ria de Aveiro, the sediment was collected with a shovel, digging about 30 cm depth, and the *Diopatra* tubes were gently removed from the sediment. In the laboratory, the animals were pushed out from the tube and examined alive. Specimens from the other localities were obtained from earlier collecting trips samplings (Table 1) and were re-examined for taxonomic confirmation. In those localities the sediment was collected with grabs, either a 0.1 m^2 Smith-McIntyre (shelf off Aveiro, Guia and Nazaré) or a 0.05 m^2 Ponar (southern coast). The samples were washed through a 1 mm mesh sieve and fixed with 4% formalin neutralized with borax. All organisms collected were sorted and identified under a stereomicroscope and then transferred for long-term storage in 70% ethanol.

Eight *Diopatra micrura* specimens from Guia, five from Ria de Aveiro and two *Diopatra* sp. specimens from Arcachon Bay, France, were collected for genetic studies and preserved in ethanol (96%).

Morphological characterisation and data analysis. In the laboratory, 88 adult specimens of *D. micrura* (five from Ria de Aveiro, nine from the shelf off Aveiro, three from the shelf off Nazaré and 71 from Guia, off the Tagus Estuary) and eight specimens of *Diopatra* sp. from France (seven from Arcachon Bay and one from Marennes Oléron, about 150 km North of Arcachon), were examined for morphological studies. Fixed specimens were measured for the width of the 10th chaetiger (without parapodia), the length of antennae and palps. Total length of complete specimens was measured. The numbers of chaetigers in complete specimens, of rings on the ceratophores, of whorls of the branchiae and of teeth in the pectinate chaetae were counted. The first chaetiger with subacicular hooks and the last chaetiger with branchiae were recorded. The colour pattern and the form of the prostomium of the species were described in live and preserved specimens. The terminology used for the prostomial appendages followed Paxton (1998). The relationship between the width of the 10th chaetiger, taken as a measure of the specimen size, and other morphological descriptors was analysed through linear regression analysis.

The morphological data of *Diopatra micrura* **sp. nov.**, and of the *Diopatra* specimens obtained from France were compared to the data presented by Rodrigues *et al.* (2009), for *D. neapolitana* and *D. marocensis*. Using the data recorded for the width of the 10th chaetiger, the number of rings in the ceratophores, the number

of whorls in the branchiae, the number of teeth in the pectinate chaetae, the first chaetiger to show subacicular hooks and the presence-absence of ventral lobe in the parapodia 5–20, a data matrix was constructed using as many specimens per species as possible (41 *D. micrura* **sp. nov.**, 35 *D. neapolitana*, 35 *D. marocensis* and 8 *Diopatra* sp. specimens obtained in France). Following normalisation of the variables, the morphological data matrix was submitted to classification, using Un-weighted Pair Group Mean Average upon the Euclidean distance matrix between specimens, and ordination, using Principal Components Analysis, with the software PRIMER v6 (Clarke & Gorley 2006).

The holotype and five paratypes of the new species were deposited in the Museu Nacional de História Natural, Lisbon (MNHN), five paratypes in the Museo Nacional de Ciencias Naturales, Madrid (MNCNM), and six paratypes in the Australian Museum, Sydney (AM). The remaining specimens (including specimens used for DNA sequencing) are kept at the Departamento de Biologia, Universidade de Aveiro, CESAM, Campus Universitario de Santiago, Aveiro (UA). The specimens for scanning electron microscopy (SEM) viewing were fixed in formalin. The specimens were dehydrated in graded ethanol series and critical point dried in a Bal-Tec CPD-030 critical point dryer, using liquid CO_2. After drying, the specimens were sputter coated with gold: palladium alloy 60:40 in a Polaron sputter coating system. SEM micrographs were taken in a Hitachi SU-70 scanning microscope.

Grain-size analysis. Sediment grain-size was analysed in the sites where *D. micrura* was collected, by wet and dry sieving following Quintino *et al*. (1989). The sediment was classified according to the median value (P_{50}), following the Wentworth scale (Doeglas 1968): very fine sand (median between 0.063–0.125 mm); fine sand (0.125–0.250 mm); medium sand (0.250–0.500 mm) or coarse sand (0.500–1 mm). The final sediment classification adopted the description "clean", "silty" or "very silty", when fines (particles with diameter below 0.063 mm) were below 5%, from 5% to 25%, and from 25% to 50%, respectively, of the total sediment, dry weight (Quintino *et al*. 1989). Sediments with more than 50% fines were classified as mud.

Genetic characterisation and data analysis. Total genomic DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer protocol. Purified DNA was aliquoted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20 ºC, until required.

About 500 bp part of the mitochondrial 16S rDNA and about 700 bp of cytocrome c oxidase subunit I (COI) were amplified by PCR. Amplification of the 16S rDNA gene was performed using the 16SarL and 16SbrH primers from Palumbi *et al.* (1991). The COI gene fragment was amplified using the LCO 1490 and HCO 2198 primers of Folmer *et al*. (1994).

Each PCR was performed in a final volume of 50 µl containing 10–100 ng of genomic DNA, 1 µM of each primers, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega) and 0.5 U Taq DNA polymerase (Promega). Amplification occurred on an MJ Mini Thermal-Cycler (Citomed). The thermal cycling parameters were: initial denaturation at 94 ºC, 3 min., followed by 34 cycles of denaturation at 94 ºC, 1 min.; primer-specific annealing temperature (49 ºC for 16S and 45 ºC for COI), 30 sec.; extension at 72 ºC, 2 min. and final extension at 72 ºC for 5 min. The amplification products were visualised, after agarose gel electrophoresis and ethidium bromide staining.

The DNA sequences determinations of 16S/COI PCR-amplified were commercially performed (STAB Vida, Portugal). The nucleotide sequence of each fragment was determined on both strands of PCR products from two independent reactions. The DNA and deduced amino acid sequence alignments were made with the Biological Sequence Alignment Editor (BioEdit v7.0.0, free software by Tom Hall, Department of Microbiology, North Carolina State University, USA). The sequences obtained for *D. micrura* **sp. nov.**, and *Diopatra* sp. from Arcachon were compared to *D. neapolitana* and *D. marocensis*, obtained in a previous study (Rodrigues *et al*. 2009). The DNA sequences were analysed with Genetyx-WIN v5.1 (Software Development, Tokyo), to determine the divergence percentage between the various *Diopatra* species, for both genes. These sequences were also analysed together in a single dataset, separately for each gene, with *Marphysa sanguinea* (Montagu 1813) as the outgroup. The dataset sequences were aligned in MEGA v4 (Tamura *et al.* 2007) with CLUSTALW, using the default alignment settings. The phylogenetic analysis were conducted using MEGA v4 (Tamura *et al*. 2007) by applying Neighbor Joining (NJ). To verify the robustness of the internal nodes of NJ trees, bootstrap analysis was carried out using 1,000 pseudo replicates.

Results

***Diopatra micrura*, sp nov.**
Figs. 1–8; Tables 1–3

Type material. *Holotype*: MNHN MB29-000166, Sta. RA4 (Nov-09) (incomplete specimen, 51 mm long (61 chaetigers), 3.3 mm wide).

Paratypes: MNHN MB29-000167, Sta NS (1); MNHN MB29-000171, Sta.RA1 (1); MNHN MB29-000168, Sta TS14B (1); MNHN MB29-000170, Sta TS23C (1); MNHN MB29-000169, Sta TS24 (1); MNCN 16.01/11627, Sta. TS1A (1); MNCN 16.01/11628, Sta.TS3A (1); MNCN 16.01/11629, Sta. TS4A (1); MNCN 16.01/11630, Sta. TS22 (1); MNCN 16.01/11631, Sta. TS25 (1); AM W36251, Sta. NS (1); AM W36252 Sta. TS13 (1); AM W36253 Sta. TS23 (2); AM W36254, Sta. TS23C (1); AM W36255, Sta. RA2 (1); DBUA-01140.01, Sta. TS1B (1); DBUA-01141.01, Sta. TS12 (1); DBUA-01142.01, Sta. TS12A (1); DBUA-01143.01, Sta. TS14C (1.); DBUA-01144.01, Sta TS23A (1).

Etymology. The striped antennae of the new species evoke the pattern of the coral snakes *Micrurus* spp., hence the name *Diopatra micrura*, **sp. nov.**

Morphological description. Length of complete preserved specimens from 1.7 to 7.8 cm, number of chaetigers from 70 to 97; width of 10th chaetiger from 0.6 to 4.5 mm without parapodia. Some incomplete specimens regenerating anterior end of body (paratype AM W36252); one specimen posterior end.

Overall colour of living specimens greenish dorsally, cream ventrally. Antennostyles and palpostyles with very characteristic transverse brown bands, 4–8 on antennae and 2–4 on palps (Figs 2A–C, 3A). Frontal lips whitish with brown pigment at base and ceratophores with brown rings (Fig 2B). Prostomium with brown pigment; area of nuchal grooves paler (Figs 2B, C). Peristomium with brown pigment (Fig. 2C, 3A), peristomial cirri cream. Additionally, anterior 10–15 chaetigers with small iridescent white spots (Fig. 2C) and following chaetigers with iridescent transverse white line (Fig. 2A). Laterally, from chaetigers 1–4 to 13–23 two brown patches, one on each side (Figs 2C, 3A). Branchiae green, parapodia cream (Fig. 2D); dorsal cirri with iridescent white spots.

In preserved individuals, the body is cream with two brown patches laterally on each segment up to chaetigers 13–23 (Fig. 3A). Lack of coloration in middle of each chaetiger forming “white” line along body (Fig. 3A). Brown pigmentation of antennae, palps, ceratophores, prostomium, frontal lips and peristomium noticed in living specimens still present.

Prostomium anteriorly rounded with subulate frontal lips (Figs 4A, B). Ceratophores of antennae and palps with proximal rings and longer distal ring, holotype with 14 proximal rings, other specimens with 12–15 rings (Figs 2A–C, 3A, 4A). Antennostyles relatively long, tapering to distal end, ending in fine point; in holotype laterals reaching chaetiger 9, median reaching chaetiger 5, in other specimens 6–13 and 4–10 respectively; palpostyles shorter, reaching chaetiger 2 in holotype, 2–4 in other specimens. Length of antennae quite variable, apparently unrelated to size of specimens (based on width of 10th chaetiger) (Fig. 5A). Sensory buds present on antennostyles and palpostyles forming 12–14 irregular longitudinal rows (Fig. 4C). Sensory buds slightly raised, with pores forming circles (Fig. 4D). In addition, randomly distributed sensory buds on ceratophores, frontal lips, upper lips, prostomium, peristomial cirrus, peristomium and branchiae (Figs 4G, H). Nuchal grooves crescentic (Fig. 4E). Peristomium as long as first chaetiger, bearing pair of peristomial cirri, about twice as long as peristomium (Fig. 3A).

First four modified parapodia (chaetiger 1 to 4) projecting laterally and slightly anteriorly, slightly longer than following non-modified, laterally projecting parapodia (Figs 4B, F). Prechaetal lobes rounded, present up to chaetigers 7–11, postchaetal lobes subulate (Fig. 4F), becoming gradually smaller towards posterior region but still distinct till end of body. Ventral lobes present on chaetiger 5 to 14–20, subulate to ovate (Fig. 2D); most distinct on chaetigers 6–15, then shifting more dorsally, forming new prechaetal lip by chaetiger 20–25. Dorsal cirri subulate, becoming more slender posteriorly; ventral cirri cirriform on first 4 chaetigers. Spiralled branchiae from chaetiger 4 in holotype, chaetigers 4 or 5 in paratypes, best developed from chaetigers 6 to 9 with 8–14 whorls, reaching to prostomium when anteriorly extended (Figs 2A, C); decreasing gradually

towards posterior end, absent from chaetiger 45 in holotype, chaetigers 32–55 in other specimens, depending on size of specimens (Fig. 5B). Branchial filaments fine and short, only slightly longer than width of branchial stem (Figs 2D, 3D).

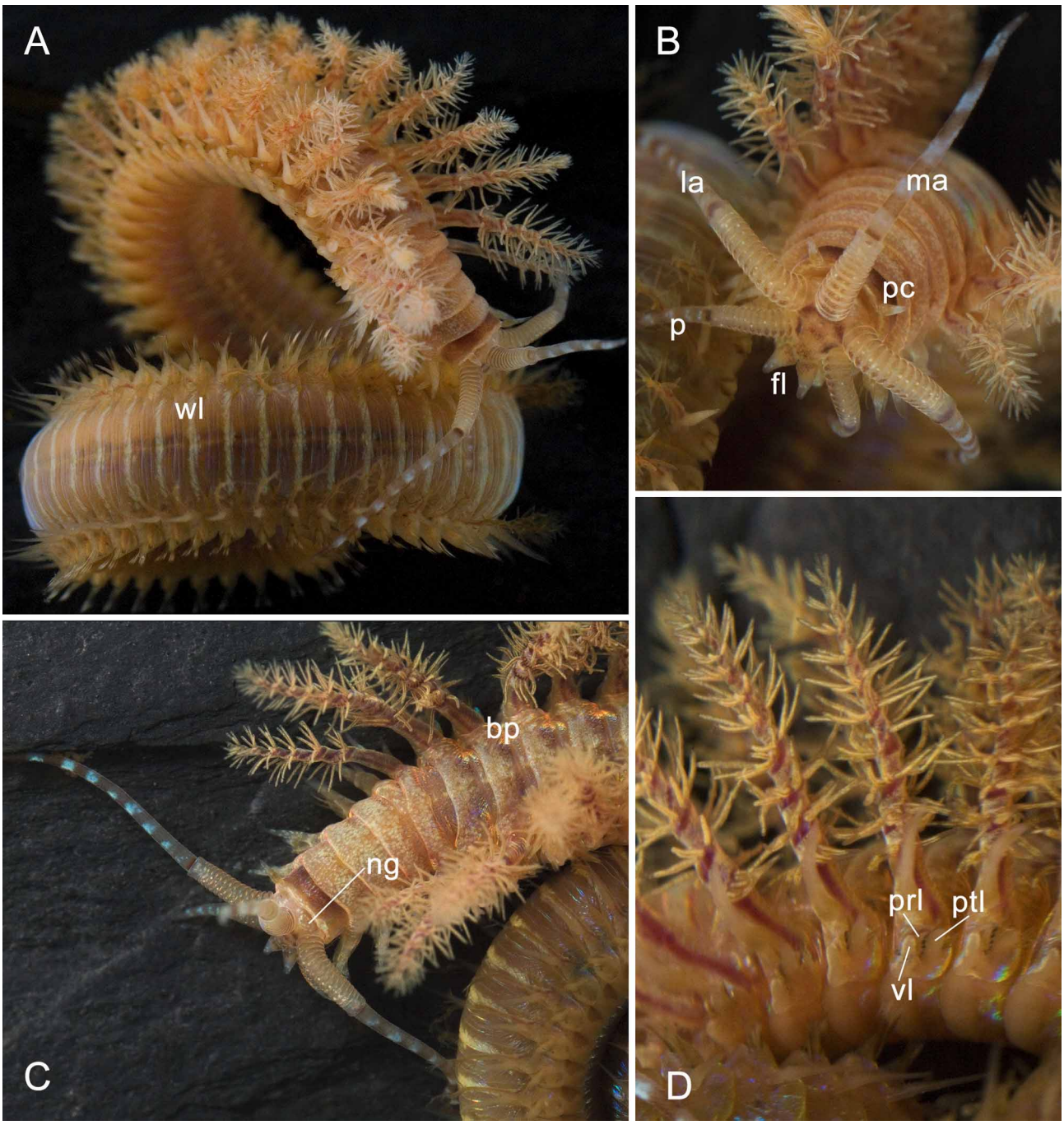

FIGURE 2. Live specimen of *Diopatra micrura* **sp. nov.**: A, general view; B, prostomium, frontal view; C, anterior end, dorsal view; D, anterior unmodified parapodia and branchiae, lateral view; (bp) brown patch; (fl) frontal lip; (la) lateral antenna; (ma) median antenna; (ng) nuchal groove; (p) palp; (pc) peristomial cirrus; (prl), prechaetal lobe; (ptl) postchaetal lobe; (vl) ventral lobe; (wl) white transverse line.

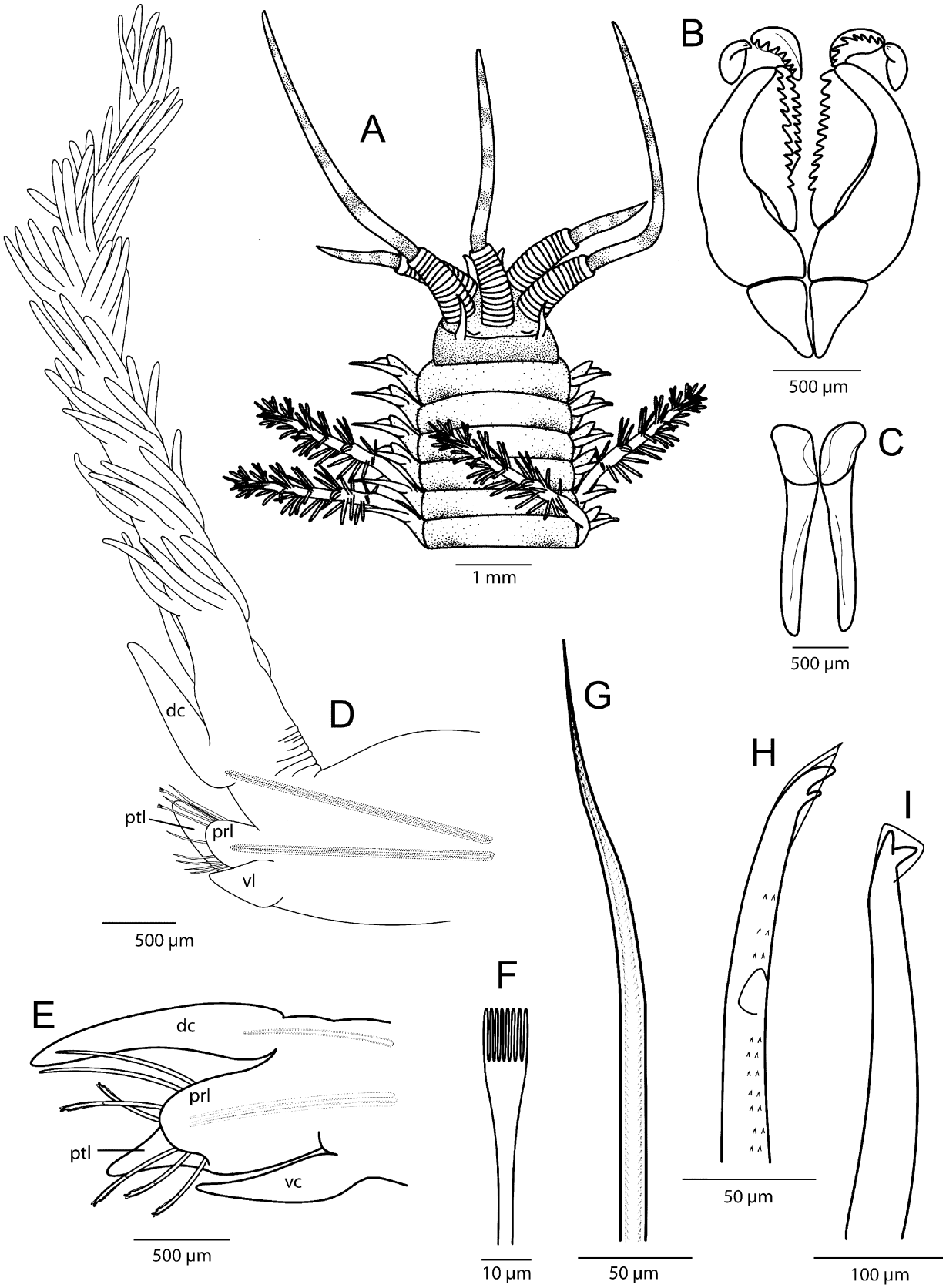

FIGURE 3. *Diopatra micrura* **sp. nov.**: A, anterior end, dorsal view; B, maxillary apparatus, dorsal view; C, mandibles, ventral view; D, parapodium of chaetiger 6, anterior view; E, parapodium of chaetiger 1, anterior view; F, pectinate chaeta; G, limbate chaeta; H, pseudocompound hook; I, subacicular hook; (dc) dorsal cirrus; (prl) prechaetal lobe; (ptl) postchaetal lobe; (vl) ventral lobe; (vc) ventral cirrus.

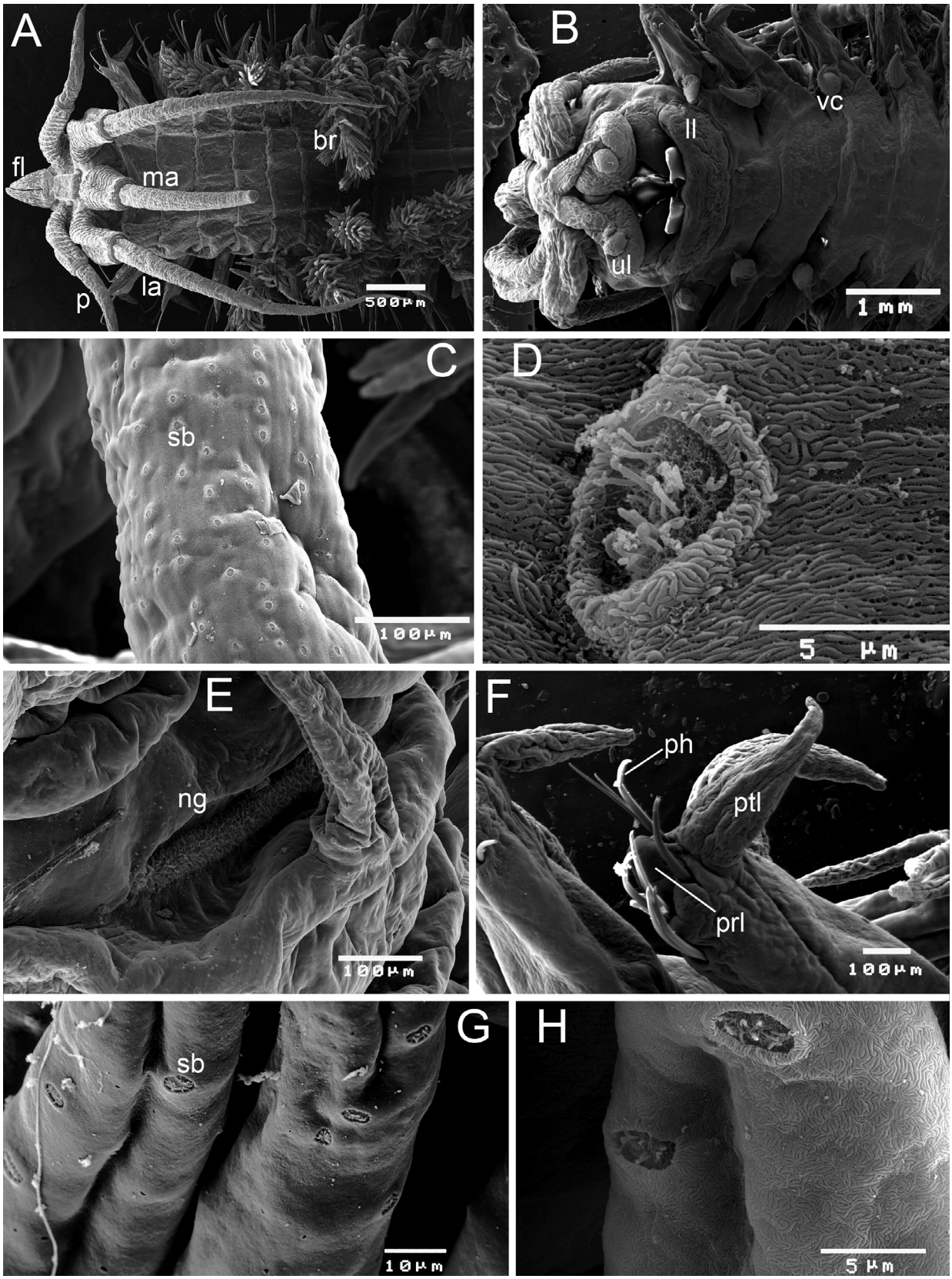

FIGURE 4. Scanning electron micrographs of *Diopatra micrura* **sp. nov.**: A, anterior end, dorsal view; B, anterior end, ventral view; C, rows of sensory buds on antenna; D, enlarged sensory bud of antennae; E, peristomium and nuchal groove area; F, modified parapodium; G, sensory buds on branchiae; H, enlarged sensory bud of branchiae; (br) branchiae; (fl) frontal lips; (la) lateral antenna; (ll) lower lip; (ma) median antenna; (ng) nuchal groove; (p) palp; (ph) pseudocompound hook, (prl) prechaetal lobe; (ptl) postchaetal lobe; (sb) sensory bud; (ul) upper lip; (vc) ventral cirrus.

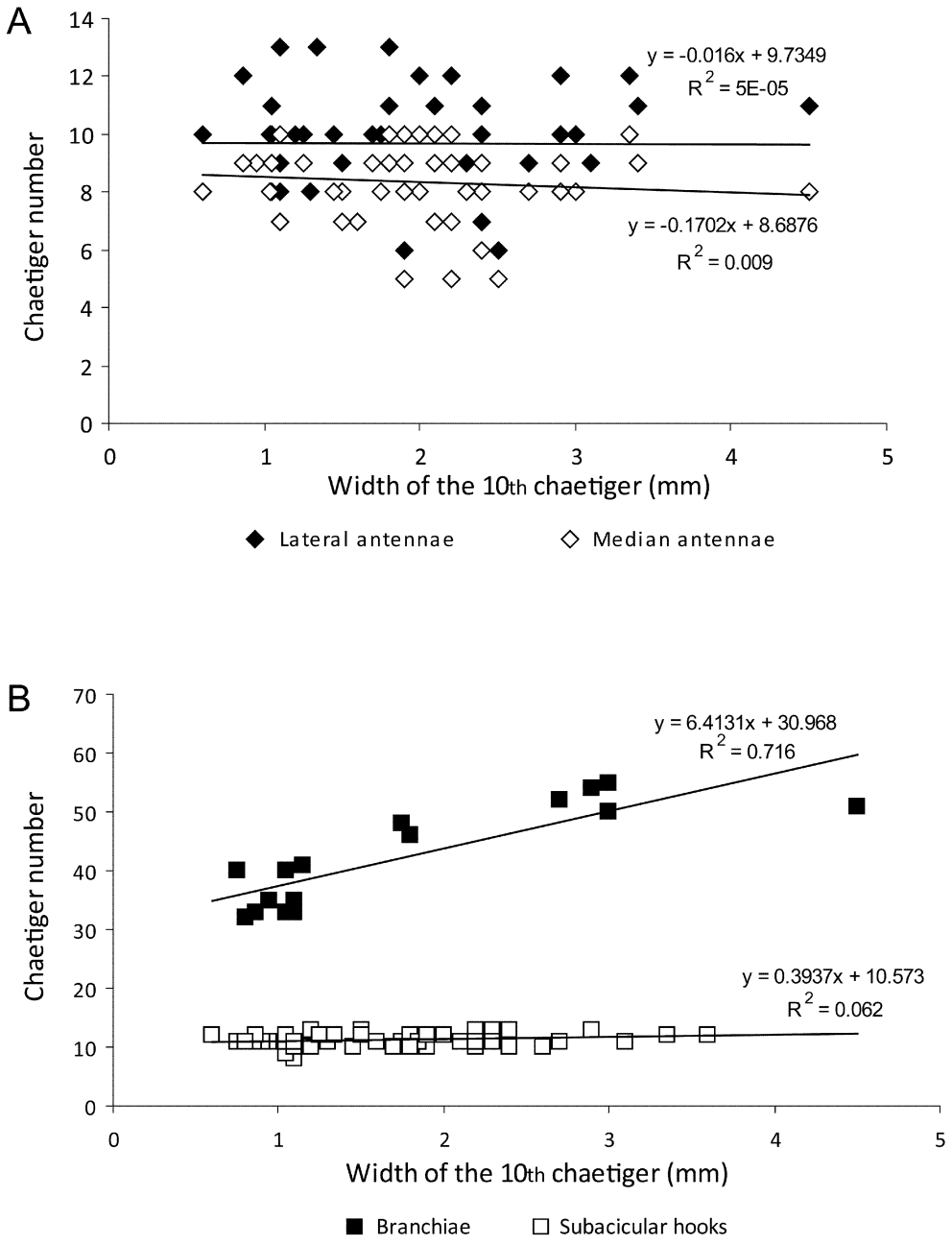

FIGURE 5. *Diopatra micrura* sp. nov.: A, relationship between body width (chaetiger 10, without parapodia) and length of lateral and median antennae. B, relationship between body width (chaetiger 10, without parapodia) and chaetigers of last branchiae and first subacicular hooks.

Modified parapodia with 1–2 slender upper limbate chaetae and 5–6 bidentate pseudocompound hooks (Fig. 3E). Hooks with moderately long pointed hoods (Figs 3H, 6A) and two rows of small spines along their shafts (Figs 3H, 6B). Remaining parapodia with limbate and pectinate chaetae (Figs 3D, 6C, D). Pectinate chaetae flat, with 5–10 long teeth, ending in slender tips (Figs 3F, 6E); limbate chaetae with narrow serrated wings, overall spiny (Figs 3G, 6F). Starting from chaetiger 11 in holotype, chaetigers 8–13 in other specimens, lower limbate chaetae replaced by 2 thick bidentate subacicular hooks with translucent guards (Fig. 3I). Slope of the regression line of start of subacicular hooks very close to nil, indicating a non-significant relationship to size of specimens (Fig. 5B).

Pygidium with two pairs of anal cirri; dorsal pair about as long as the last six chaetigers, ventral pair about as long as the two last chaetigers.

Mandibles (Fig. 3C) weakly sclerotised, with slender shafts and strongly calcified cutting plates. Maxillae moderately sclerotised (Fig. 3B). Maxillary formula (based on 9 paratypes): Mx I= 1+1; MxII = 8–10 + 8–11; Mx III = 8–11 + 0; Mx IV = 5–8 + 7–11; Mx V = 1 + 1.

Tube characteristic of genus, cylindrical with soft inner secreted layer and outer layer of debris, fragments of sea grass, algae and shells.

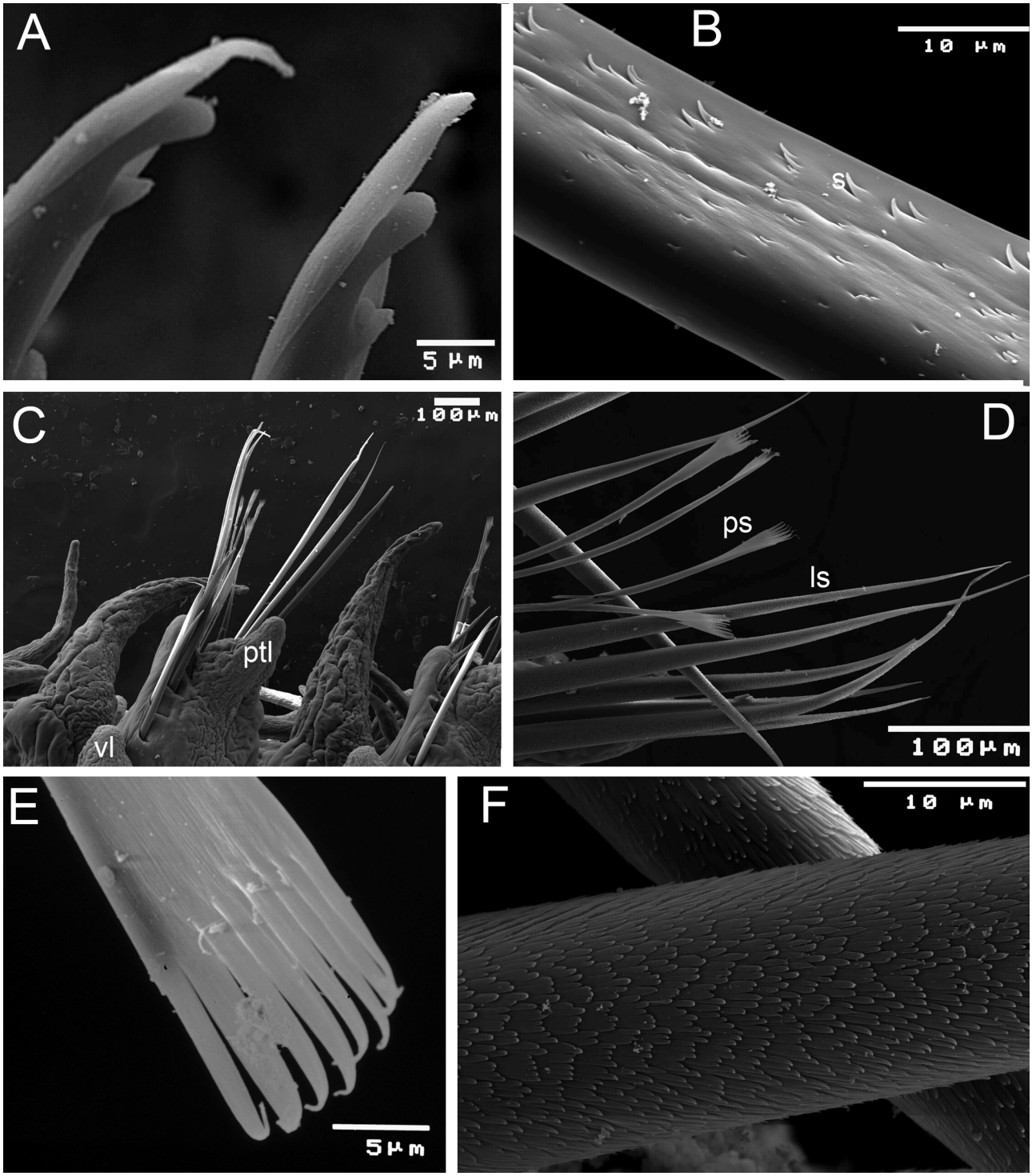

FIGURE 6. Scanning electron micrographs of *Diopatra micrura* **sp. nov.**: A, distal ends of pseudocompound hooded hooks; B, spines of pseudocompound hooded hook; C, parapodium of chaetiger 9; D, pectinate and limbate chaetae of parapodium 13; E, pectinate chaeta; F, spines of limbate chaetae; (s) spines; (ls) limbate chaeta; (ps) pectinate chaeta; (ptl) postchaetal lobe; (vl) ventral lobe.

TABLE 2. Intraspecific variability of the most important morphological characters of *Diopatra micrura* **sp. nov.** (SD = Standard deviation, N = number of individuals observed).

Character	Range	Mean	SD	N
Length, complete preserved specimens (cm)	1.7–7.8	5.4	3.25	3
Number of chaetigers, complete specimens	70–97	86.0	14.18	3
Width of 10th chaetiger without parapodia (mm)	0.6–4.5	1.90	0.78	77
Lateral antennophores (number of rings)	12–15	14.42	0.75	85
Median antennophore (number of rings)	12–15	13,46	0,91	83
Palpophores (number of rings)	12–15	13.36	0.93	87
Lateral antennae (reaching chaetiger)	6–13	9.64	1.71	66
Median antenna (reaching chaetiger)	4–10	8.25	1.43	67
Palps (reaching chaetiger)	2–4	2.26	0.48	72
Peristomial cirrus/peristomium (length ratio)	1.5–2.8	1.84	0.30	63
First branchiae (chaetiger)	4–5	4.54	0.5	89
Last branchiae (chaetiger)	32–55	42.82	8.34	18
Branchial whorls (maximum number)	8–14	10.92	1.79	75
First subacicular hooks (chaetiger)	8–13	11.24	1.08	57
Last prechaetal lobes (chaetiger)	7–11	9.36	1.16	42
Last ventral lobes (chaetiger)	14–20	16.83	1.80	37
Pectinate chaetae (number of teeth)	5–10	7.00	0.98	45

Remarks. The intraspecific variability of the major morphological characters of *Diopatra micrura* is summarised in Table 2 and the comparison with other European *Diopatra* species in Table 3.

The multivariate analysis of the morphological data is shown in Figure 7. The groups of individuals belonging to the various species form distinct clusters (Fig. 7, upper graph), represented by well isolated clouds of points in the ordination diagram (Fig. 7, lower graph). The PCA axis 1 and 2 comprehend 91.3% of the total variance. *Diopatra neapolitana* opposes *D. marocensis* on the ordination axis 1, with *D. micrura* occupying a transition position, on the positive pole of axis 2. Most of the *Diopatra* specimens from France form a distinct cluster, isolated in the negative pole of axis 2 but closer to *D. marocensis*. This cluster includes five specimens from Arcachon Bay and a single specimen from Marennes Oléron (Fig. 7). Nevertheless, two specimens from the Arcachon Bay are plotted together with the cluster of *Diopatra neapolitana*, indicating the coexistence of at the least two species in this Bay. The morphological descriptors most strongly correlated with PCA axis 1 were the number of branchiae whorls (r = - 0.92), the number of rings in the ceratophores (r = - 0.90) and the presence-absence of ventral lobe in the parapodia 5–20 (r = - 0.85). The chaetiger where the subacicular hooks start (r = - 0.72), the width of the 10th chaetiger (r = - 0.70) and the number of teeth in the pectinate chaetae (r = - 0.53), were the variables strongly correlated with PCA axis 2, the latter especially related to the *Diopatra* sp. individuals from Arcachon Bay (Fig. 7).

Distribution and habitat. *Diopatra micrura* occurs along the western and southern Portuguese coast. Specimens were collected in Ria de Aveiro, near the mouth, intertidally and on the adjacent shelf area (A), on the shelf off Nazaré (B), in Guia, off the Tagus Estuary (C), and near the Guadiana river mouth (D) (Fig. 1).

The species seems to have a preference for fine sand and shallow waters. In the shelf area off the Tagus estuary, it was found in 22 of the 30 sites comprising the annual monitoring program of this area, carried out since March 1994. *Diopatra micrura* has been found in every annual sampling campaign, in sites ranging from 40 to 60 metres depth, on fine and very fine sand, with fines content up to 25% of total sediment. In the Aveiro shelf, *D. micrura* was found in 8 of 22 sites sampled in 2002, always close to 15 metres depth and in fine and very fine sand with less than 5% fines content. In the Nazaré shelf, the species was found at 37 metres depth, on fine sand with 7% fines. On the southern coast, it was found in fine and very fine sand with less than 5% fines, ranging from 4 to 10 metres depth. Finally, in Ria de Aveiro the species was found in the intertidal region, together with *D. marocensis* and *D. neapolitana*, in very fine sand with close to 25% fines content.

TABLE 3. Comparison of morphological descriptors (mean ± standard deviation) measured in *Diopatra micrura* sp. nov., *D. neapolitana* and *D. marocensis* from Portuguese waters and in the Arcachon specimens (presumably *Diopatra* sp. A mentioned in Berke *et al.* 2010). Range between brackets.

Character	*D. neapolitana* (Rodrigues et al., 2009)	*D. marocensis* (Rodrigues et al., 2009)	*D. micrura* sp. nov.	Specimens from France	
				Diopatra sp.	*D. neapolitana*
Colour (living specimens)	greenish	pinkish	greenish	-	-
Length, complete preserved specimens (cm)	36.39 ± 13.50	8.93 ± 1.98	5.4 ± 3.25	-	-
Number of chaetigers, complete specimens	269.20 ± 31.16	141.69 ± 22.8	86.00 ± 14.18	-	-
Average width of 10th chaetiger (mm)	7.08 ± 1.68	2.97 ± 0.66	1.90 ± 0.78	7.08 ± 0.66	5.25 ± 1.06
Frontal lips (shape)	subulate	subulate to ovate	subulate	subulate	subulate
Nuchal grooves (shape)	rounded	crescentic	crescentic	rounded	rounded
Sensory buds on antennae and palps (numbers of rows)	20-22	16-18	12-14	-	-
Ceratophores (number of rings)	15-16	6-9	12-15	9-11	14-16
First branchiae (chaetiger)	4.46 ± 0.51 (4-5)	4.14 ± 0.36 (4-5)	4.54 ± 0.5 (4-5)	4.33 ± 0.52 (4-5)	4.5 ± 0.71 (4-5)
Last branchiae (chaetiger)	64.40 ± 3.53 (56-70)	33.77 ± 2.96 (26-38)	42.82 ± 8.34 (32-55)	52.67 ± 4.73 (49-58)	51.0 ± 4.24 (48-54)
Branchial whorls (maximum number)	14-18	6-9	8-14	9-12	15
First subacicular hooks (chaetiger)	19-25	13-15	8-13	15-17	19
Last prechaetal lobes (chaetiger)	15-20	6-10	7-11	16-19	18-20
Ventral lobes	present	absent	present	absent	present
Pectinate chaetae (number of teeth)	5-10	11-20	5-10	25-32	6-9
Specimens (number examined)	35	35	88*	6	2
Locality	Aveiro	Aveiro, Guia	Aveiro, Guia	Arcachon Bay, Marennes Olérons	Arcachon Bay
Habitat	Intertidal	Intertidal/Subtidal	Subtidal/Intertidal	Intertidal	Intertidal

* Total number of specimens. Check Table 2 for the exact number of specimens observed for each descriptor.

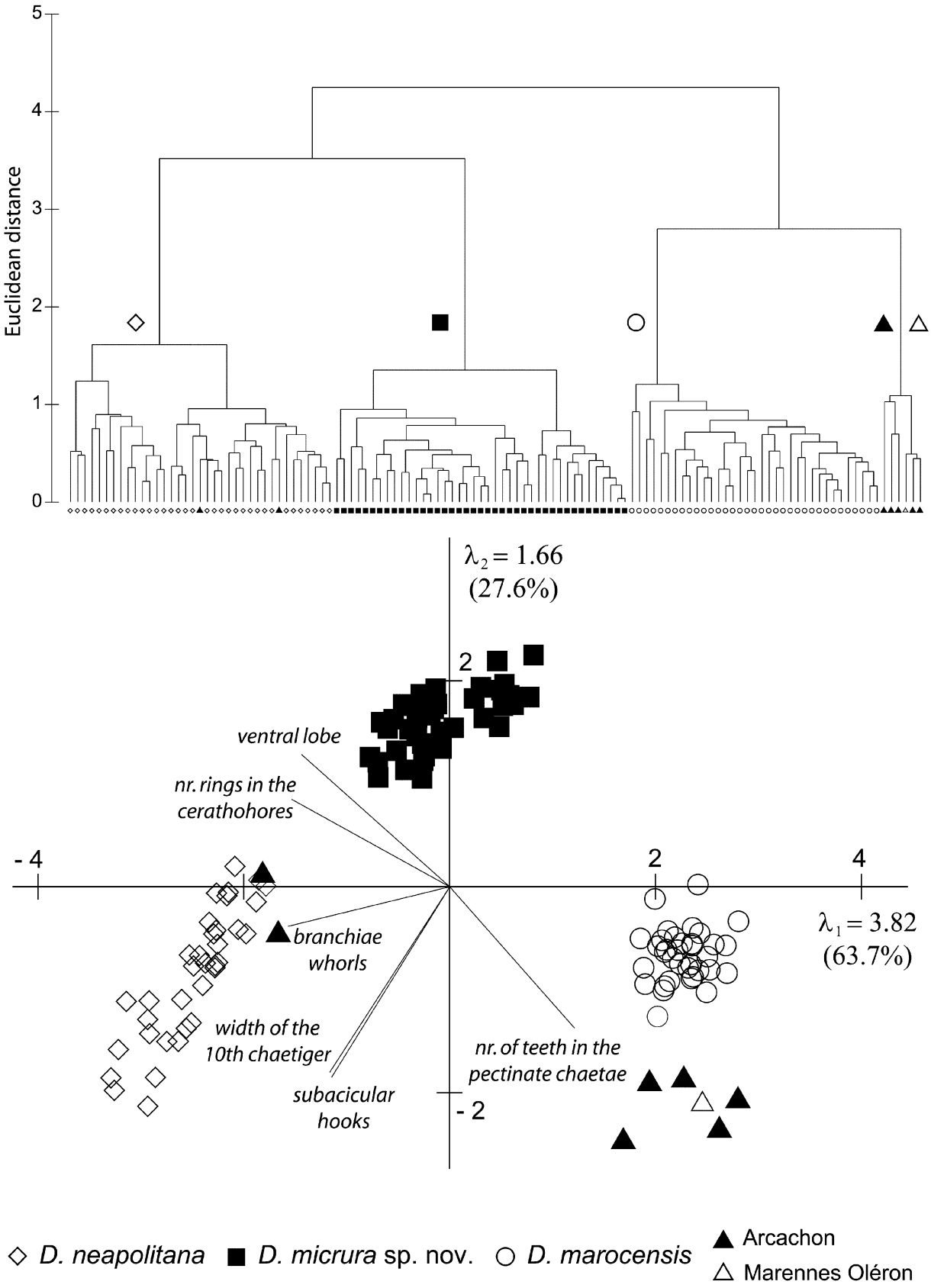

FIGURE 7. Classification (upper graph) and ordination (lower graph) analysis of specimens of European *Diopatra* species, according to morphological descriptors. Most of the specimens obtained in France (Arcachon Bay and Marennes Oléron) form an isolated cluster, corresponding to a fourth species, but also include individuals belonging to *D. neapolitana*.

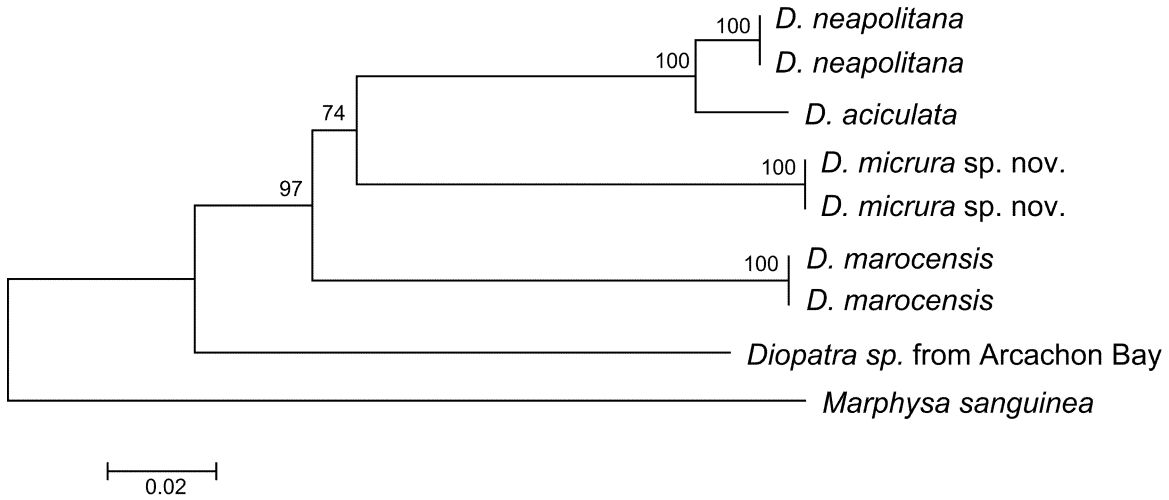

FIGURE 8. Phylogenetic analysis of the data set containing the 16S and COI sequences of *Diopatra* species. Numbers near the nodes indicate the percent bootstrap values. The branch length indicator represents 0.02 substitutions per site.

Genetic analysis. A 702-bp COI fragment and a 525-bp 16S fragment were successfully obtained from 14 individuals of *D. micrura*. COI and 16S nucleotide sequences from *D. micrura* **sp. nov.**, were deposited at EMBL database, under the accession numbers: 16S – GQ456163 and COI – GQ456161 and GQ456162.

For the 16S gene, all individuals displayed identical nucleotide sequence but in the case of the COI gene, one individual from Ria de Aveiro presented a base alteration, at position 276, where a nucleotide adenine was replaced by a thymine (ATA to TTA), corresponding to an amino acid alteration (methionine to leucine). All specimens sampled on the shelf off the Tagus Estuary shared the same nucleotide sequence.

The percentage of nucleotides divergence of the 16S and COI genes between *D. micrura* and *D. marocensis* was 15% and 17%, respectively (nucleotide substitution). For *D. micrura* and *D. neapolitana*, the divergence was 16% for COI and 12% for 16S. For COI, deduced amino acid sequence comparison between the species revealed that *D. micrura* differs from *D. marocensis* in six amino acids and from *D. neapolitana* in two amino acids, for one haplotype, and in three for the other, revealing 2.59% and 1.08% of divergence, respectively. The majority of the differences in nucleotides between *D. micrura* and those two species occurred on the third position of the codon and therefore corresponded to silent alterations.

Comparing COI and 16S genes of *Diopatra* sp. from Arcachon Bay with *D. neapolitana*, *D. marocensis* and *D. micrura*, the percentage of nucleotides divergence varied between 17% and 19% in the case of the COI and between 16% and 19%, for 16S gene (nucleotide substitution). The phylogenetic analysis from both genes (Fig. 8) separates the *Diopatra* species into four clades.

Discussion

The new species, *Diopatra micrura*, was found on the western and the southern coast of Portugal, in fine or very fine sand with less than 30% of fines content, from the intertidal region up to 50 metres depth. *Diopatra micrura* coexists with other *Diopatra* species, namely *D. neapolitana* and *D. marocensis* but it is much less common and was never recorded in densities as high as those of the other two species. This study also showed the coexistence between *D. neapolitana* and *Diopatra* sp. in the Bay of Arcachon, from intertidal specimens collected in 2009, contrary to the opinion expressed by Berke *et al.* (2010) who set the Northern limit distribution of *D. neapolitana* on the Spanish French border.

The presence of *D. micrura* off the Tagus Estuary, on the western coast of Portugal, can be traced back as far as 1994, where the species has been regularly recorded in monitoring samples taken yearly. In that same coastal area, *D. marocensis* has shown an increase in density and distributional area over the last five years (Rodrigues *et al.* 2009), but this has not, so far, excluded *D. micrura*. This study shows that *D. micrura* can be

distinguished from its European congeners by morphological and genetic characteristics and proposes a key to the European species of *Diopatra*.

Diopatra micrura is most closely related to *D. neapolitana*, a species with which it occurs sympatrically in Ria de Aveiro. Both species possess ventral lobes on parapodia 5–20. These lobes have only been observed in *D. monroviensis* Augener, 1918 from West Africa and in *D. aciculata* Knox & Cameron, 1971 from Australia. The latter is morphologically very similar to *D. neapolitana* but shows some genetic isolation as was discussed by Rodrigues *et al*. (2009). *Diopatra micrura* can easily be distinguished from the other species by its striped antennostyles and palpostyles, crescentic rather than rounded nuchal grooves, much smaller adult size and more anterior start of the subacicular hooks. Furthermore, there are differences in the construction of the tubes. The characteristic tubes of *D. monroviensis* have a thick outer layer of sand with even thicker ridges every centimetre or so, while those of the other three species lack the ridges and have also some fragments of seagrass, algae and shells attached.

In the molecular studies of the 16S and COI genes, all individuals of *D. micrura* displayed an identical nucleotide sequence for the 16S gene but, in the case of the COI gene, one individual from Ria de Aveiro presented a base alteration at position 276. This corresponded to a replacement of adenine by thymine, and an amino acid alteration occurred. However as these amino acids belong to the same chemical group (Stryer 1999) the sequences are translated in proteins of the same family that will have the same function.

The phylogenetic relationship analysis of the European *Diopatra* species revealed four clades, representing four distinct species of *Diopatra*, emphasising the validity of *D. micrura* as a distinct species. The mitochondrial genes, COI and 16S rRNA, are considered conserved genes, but the relative nucleotide divergence that we obtained between the four *Diopatra* species – averaging 17.5% and 15.4% respectively - is usual among different species of polychaetes. In fact, in the case of 16S rRNA, in the dorvilleid genus *Ophryotrocha* the mean sequence divergence is 12% (Dahlgren *et al.* 2001); in the syllid genus *Autolytus* it is about 21% (Nygren & Sundberg 2003) and within the *Palola* genus the mean divergence is 12.4% (Schulze 2006). In the case of COI, sequence divergence in the terebellid genus was 20% for two *Loimia* species and 19% for two *Amphitrite* species (Schulze 2006) and for the *Palola* genus, the mean divergence is 14.5% (Schulze 2006).

The multivariate analysis of morphological descriptors showed a very good separation between the groups of individuals from different species and allows similar conclusions regarding the validity of the four European species of *Diopatra*, for which the following key is proposed:

1 Antennae with transverse brown bands; parapodia 5–20 with ventral lobes; 12–16 rings on ceratophores 2
- Antennae without transverse brown bands; ventral lobes absent; 6–11 rings on ceratophores 3
2 Antennae with 4–8 transverse brown bands (Fig. 2A–C), small species, up to 10 cm long, 4.5 mm wide; subacicular hooks starting from chaetiger 8–13 ..... *D. micrura*, **sp. nov.**
- Antennae with single median brown band; large species, up to 40 cm long, 9 mm wide; subacicular hooks starting from chaetiger 19–25 ..... *D. neapolitana*
3 Dorsum with mid-dorsal brown patch, forming line along anterior part of body; nuchal grooves crescentic; parapodia with single postchaetal lobes; pectinate chaetae with 11–20 teeth ..... *D. marocensis*
- Dorsum without pigment; nuchal grooves rounded; parapodia 1–5 with double postchaetal lobes; pectinate chaetae with 25–32 teeth..... *Diopatra* sp. from Arcachon Bay

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ORIGINAL ARTICLE

Reproductive biology of *Diopatra neapolitana* (Annelida, Onuphidae), an exploited natural resource in Ria de Aveiro (Northwestern Portugal)

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Abstract

Diopatra neapolitana Delle Chiaje, 1841 (Annelida, Onuphidae) is an important economic natural resource in Ria de Aveiro (northwestern coast of Portugal) and throughout Europe. The species is intensively harvested for use as fresh bait. However, there is only limited knowledge about its life cycle derived from a previous study in Mediterranean Sea. Reproduction and development patterns are known to vary biogeographically, making it important to base management decisions on locally appropriate information. This work examines reproduction patterns for populations from the Eastern Atlantic, which have not previously been assessed, with an eye towards drawing Atlantic–Mediterranean comparisons and informing local management strategies. The study was conducted from May 2007 to April 2009 in Ria de Aveiro. The reproductive biology of *D. neapolitana* was described from the proportional variation of worms with gametes in the coelom and from the progression of the oocyte diameter. Individuals with gametes inside the coelom were found all year round, but the peak reproductive period occurred between May and August, when almost all individuals had gametes in the coelom and females contained more oocytes than at any other time of the year. The overall male:female ratio was close to 1:1 and the oocyte diameter ranged from 40 to 240 μm. *In vitro* fertilization was performed and the results compared to other studies. Based on the present results, some protection measures are suggested to implement a sustainable exploitation of the species.

Introduction

The polychaete *Diopatra neapolitana* Delle Chiaje, 1841 (Onuphidae) inhabits intertidal mudflats and shallow subtidal transitional waters. The geographical distribution records indicate that it is a cosmopolitan species distributed throughout the Mediterranean (Gambi & Giangrande 1986; Dagli *et al.* 2005), the Red Sea (Fauvel 1923) and the Eastern Atlantic (Fauvel 1923; Lourido *et al.* 2008) and Indian Oceans (Wehe & Fiege 2002). However, in regions of the world where careful genetic and morphological analysis has been conducted, it was shown that *D. neapolitana* harbors multiple species. In Europe, four species of *Diopatra*, *D. neapolitana*, *Diopatra marocensis*, *Diopatra micrura* and *Diopatra* sp. (not yet described) were identified and distinguished morphologically using characters that have not been used previously (Pires *et al.* 2010). Such analysis could be applied in other regions, in particular the Red Sea and Indian Ocean.

The species inhabits a tube, has a preference for sediments with mud or a mixture of mud and sand, and grows to about 60 cm (Fauvel 1923; Gambi *et al.* 1998; Dagli *et al.* 2005; Rodrigues *et al.* 2009). The tube consists of a secreted layer, to which sand particles, fragments of solid parts from other animals, such as shells, and algae attach to form a compact tube.

Larval development in the Onuphidae is dependent on yolk reserves, with some species being lecithotrophic, feeding only after settlement, and others having a direct development (without larval stages) (Blake 1975; Giangrande 1997). Conti *et al.* (2005) report that *D. neapolitana* releases eggs and sperm into the water column and Bhaud & Cazaux (1987) that it produces planktonic lecithotrophic larvae. Although the spawning of this species has never been observed in nature, artificial fertilization and culture of the larvae was reported by Bhaud & Cazaux (1987) and Conti & Massa (1998), who described several developmental phases. These authors showed that the larvae were lecithotrophic and free-swimming.

This species is collected to be sold as fish bait and this activity can be locally intense and economically important (Gambi *et al.* 1994; Conti & Massa 1998). A previous study in Ria de Aveiro, Northwestern Portugal, where the present study was undertaken, indicated an annual harvest of 45,000 kg, valued at over € 325,000 (Cunha *et al.* 2005). According to Portuguese legislation, bait collection is only allowed by hand gathering or with restricted gear, such as a hoe, operated by licensed personnel (Portuguese legislation: Portaria no 144/2006 2006). No other legislation exists for the Ria de Aveiro and no management or conservation efforts are currently being developed for this species. Its reproductive biology is relatively unknown, as the only field work ever done on this subject was carried out in the Eastern Mediterranean Sea by Dagli *et al.* (2005).

The present study focuses on the gametes' characteristics, the larval development, the reproductive period, and the sex ratio of the population of *D. neapolitana* in Ria de Aveiro. Understanding these life history aspects is important for management and conservation efforts aimed at a sustainable exploitation of the species.

Material and methods

Study area and sampling

This study was conducted in Ria de Aveiro, Northwestern Portugal (40°40′01.6″ N, 8°41′39.5″ W; Fig. 1). Ria de Aveiro is a shallow estuarine water system, receiving water from several rivers (Fig. 1), with the Vouga River accounting for more than 50% of the freshwater input, resulting in a complex system of bays, channels and extensive intertidal sand and mud flats (Dias *et al.* 1999).

Diopatra neapolitana specimens were collected intertidally, monthly from May 2007 to April 2009 with a shovel, at up to 30 cm depth. At least 50 specimens were collected randomly, each month, at the study area.

Laboratory procedures

In the laboratory worms were individually removed from their tubes and washed in sea water. Each specimen was partly dissected to search for the presence of gametes and then fixed in 70% ethanol. Fixed specimens were measured for width at the 10th chaetiger (without parapodia). Total length was measured in entire specimens (about 4% of individuals). These morphological variables were only measured in individuals that were not seen to be regenerating.

The oocytes were extracted from females by dissection of the body cavity. The diameter of each oocyte was measured under a stereomicroscope (resolution 50×) using an ocular micrometer (precision of 0.01 mm). The diameter of 100 oocytes was measured for each female. Different numbers of females were collected each month. During the periods with a larger number of mature individuals (April–August) oocytes were measured in at least 12 females. In the remaining study period, oocytes were measured in all the females collected, as their number was below 12. In some cases, only two to four females with oocytes in the coelom cavity were sampled. In total, oocytes from 332 females were measured. To count the total number of oocytes per female, only complete specimens were used – 12 in total. These were collected between May and December. During the study period, fresh sperm in sea water was observed under a microscope (resolution 1000×).

Fertilization *in vitro*

Specimens were collected from the study area and kept in the laboratory for at least 2 months. They were maintained at 22 °C and at a salinity range of 30–35. Salinity was measured with a hand-held refractometer and expressed using the practical salinity scale. To study larval development, artificial fertilization was performed following the method described by Conti & Massa (1998) for *Diopatra neapolitana*. Females and males were cut laterally and left in separated dishes with sea water for 10–15 min to release the eggs and sperm. A portion of sperm was collected and added to the oocytes. The fertilized eggs were cultured at 22 °C and 30–35 salinity. Sea water was changed daily.

The larval development observed in this study was analyzed following the descriptions of Bhaud & Cazaux (1987) and Conti & Massa (1998).

Once settled, the larvae were fed with homogenized cockles. Four days after fertilization, in the metatrocophore phase, the larvae were moved to an aquarium with fine sediment. The study of larval stages was carried out under an optical microscope.

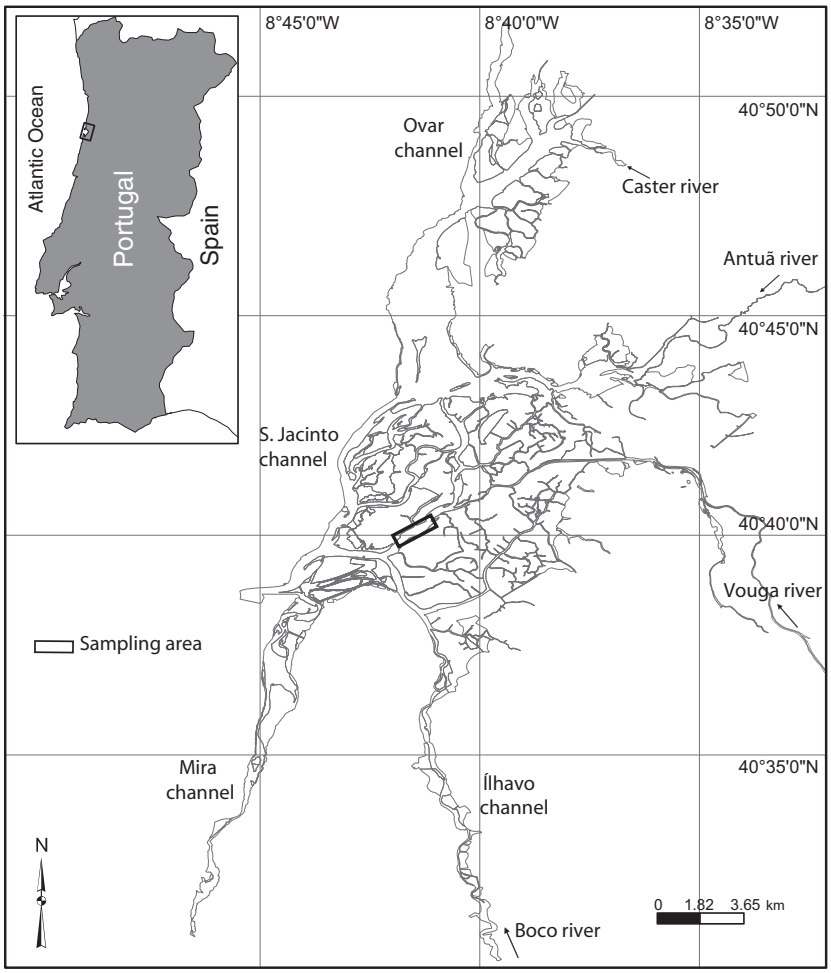

Fig. 1. General view of Ria de Aveiro, Portugal, showing sampling area.

Data analysis

The relationship between total length (L) and the width of the 10th chaetiger (W) was studied using second-order polynomial simple regression analysis. This relationship was established from 46 complete individuals, collected over the entire study period, according to the function $L = a + b_1W + b_2W^2$, forcing the model through the origin ($a = 0$). SPSS software (version 17) was used to test the overall significance of the model (*F*-test) and of the second-order regression coefficient (b_2, *t*-test). The total expected body length of broken specimens was then determined from the measured width of the 10th chaetiger, using the regression function. This relationship was used to determine the expected shortest length of mature individuals.

The mean oocyte diameter (MOD) was calculated per female and per month, and its correlation with total length was assessed using the Pearson coefficient. The variance of oocyte diameter in the period of gametogenesis inactivity (November–January) was statistically compared (*F*-test) with the period of gametes production (March–October).

Results

Relationship between total length and width of the 10th chaetiger

Entire mature specimens ranged in size from 24 to 725 mm, with the width of the 10th chaetiger varying between 1.9 and 10.88 mm, respectively (Fig. 2). All observed specimens, entire or incomplete, had a 10th chaetiger width of between 1.9 and 13 mm. The regression function relating the body length of the specimens (L, in mm) to the width of the 10th chaetiger (W, in mm) was statistically significant ($F = 1081.5$; $P < 0.0001$) and was given by the expression $L = 17.955\ W + 4.209\ W^2$. The regression coefficient associated with W^2 ($b_2 = 4.209$) was also found to be significantly different from zero, validating the second-order polynomial ($t = 6.945$; $P < 0.0001$). Under this

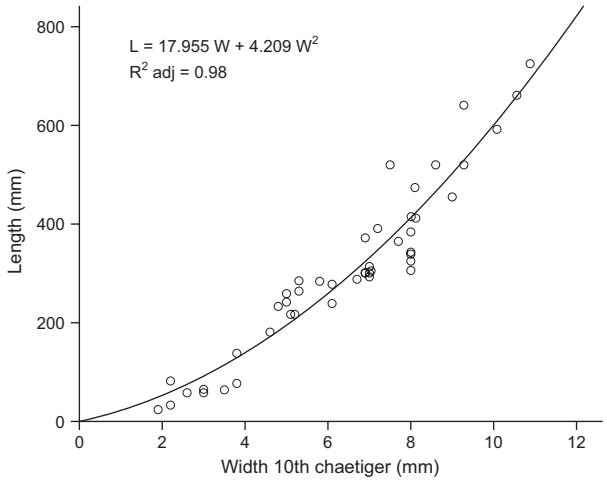

Fig. 2. Relationship between total length (L) and width of the 10th chaetiger (W).

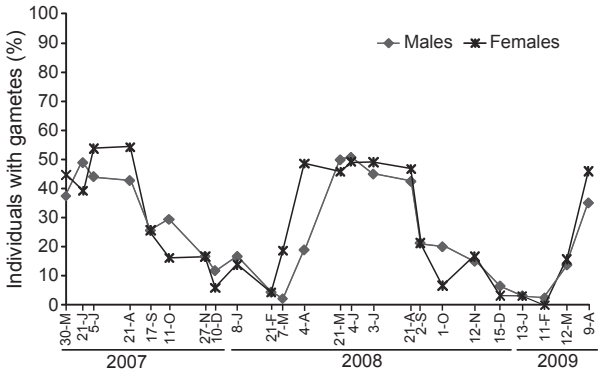

Fig. 3. Temporal development of males and females given as a percentage of the total number of specimens analyzed monthly. Only individuals with gametes were considered.

regression model, the width of the 10th chaetiger explained 98% of the total length variance ($R^2_{adj} = 0.98$). This regression function was used to estimate the total length of broken specimens. The smallest female observed to be carrying oocytes had W = 4.2 mm, corresponding to an estimated body length of 149.7 mm. The smallest male with sperm in the coelom had W = 4.0 mm, corresponding to an expected body length of 139.2 mm.

Table 1. Mean number of oocytes (with the standard deviation; SD) in complete females. The number of females analyzed in each month is shown in parentheses.

months	No. oocytes	SD
May (2)	1,821,846.5	140,700.81
June (3)	453,885.7	90,239.58
August (3)	73,131.5	7252.79
September (2)	30,880.5	11,716.05
October (1)	20,190	–
December (1)	20,818	–

Reproduction of *Diopatra neapolitana*

The presence/absence of gametes was analyzed in 1163 specimens, of which 320 were males, 332 females and 511 undetermined (with no gametes in the coelom). No external morphological differences were noticed between males and females. However, during the main reproductive period males turned a cream color and females became greenish, mainly due to the gametes in the coelom.

The overall male:female sex ratio was close to 1:1 from April to September. For the other months, very few individuals with gametes were captured and the sex ratio was not determined.

The reproductive cycle of *D. neapolitana* can be inferred from the proportional variation of worms with gametes in the coelom, from the development of the size of oocytes and from the number of oocytes in complete females (Figs 3 and 4; Table 1). Individuals with gametes inside the coelom were always found, but the percentages of males, females and of individuals without gametes varied (Fig. 3) and showed a consistent pattern in the two consecutive years. In February 2008 and February 2009, a single specimen with oocytes and a single specimen with sperm were found, respectively, whereas in April–August a larger proportion of individuals with gametes (varying from 39.22–54.29% in females and 35.14–50.0% in males) (Fig. 3) were found.

The smallest oocyte found in a female's coelom had a diameter of 40 μm and the largest a diameter of 240 μm, with the mean for all specimens being 164.4 ± 40.8 μm. Small oocytes (<140 μm) were present in almost every month. The number of small oocytes reached a peak in March and April, decreasing until September. Oocytes were absent in some autumn/winter months (October, November and December) (Fig. 4). The decrease in the number of small oocytes paralleled the increase of larger oocytes (Fig. 4). The mean oocyte diameter was not correlated with the size of the females, measured by the width of the 10th chaetiger ($r = -0.011$; $P > 0.05$). Mean oocyte diameter increased rapidly from March to May, and continued to increase slowly until January (Fig. 4). The variance in oocyte size was significantly larger from March to October ($s^2 = 1354$) than during the winter months, from November to January ($s^2 = 264$; $F = 5.1$; $P < 0.001$). This can be appreciated in Figs 4 and 5.

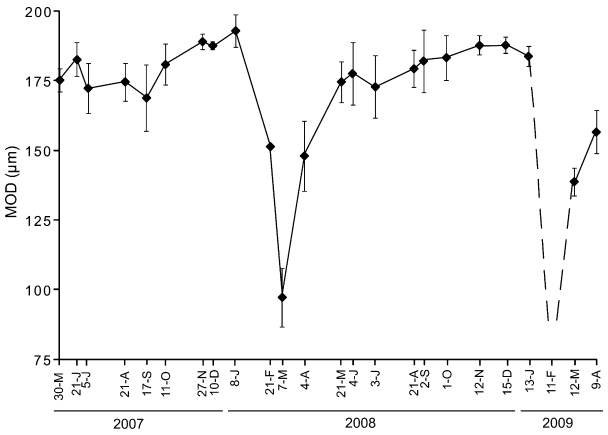

Fig. 4. Evolution of the mean oocyte diameter (MOD, μm), during the study period. No specimens with oocytes in the coelom were obtained in February 2009. The bars represent the standard deviation.

Females from November to January contained mainly large oocytes of between 140 and 240 μm (Fig. 5). Nurse cells were observed in oocytes with a diameter of up to 160 μm (Fig. 6A). They were attached to the immature oocytes with two strings measuring up to 230 μm in length (mean = 177.5 ± 35.4 μm) and containing up to 39 cells (mean 29.4 ± 4.5 μm) 12 μm in diameter. Oocytes larger than 160 μm did not have nurse cells attached (Fig. 6B).

Sperm had a spherical, short and rounded head with a long tail and were grouped in capsules in the coelom. When sperm were observed under the microscope, between May and August, the majority of the males contained spermatozoa with a mobile flagellum, moving actively in sea water. From October to January, spermatozoa had tails but reduced mobility. Sperm were immobile during the other months.

The first chaetiger with gametes varied. In females where the oocytes were observed they were located between the 35th and the 70th chaetiger. In males, sperm were found from chaetigers 50 to 70. The mean location of the chaetiger where gametes first appeared was 52.7 ± 8.6 for oocytes and 59.3 ± 7.3 for sperm. No significant correlation was found between the first chaetiger bearing gametes and the size of the individuals (r = 0.01).

In May and June, the months where it was possible to count the total number of oocytes per female (complete females), females had the highest number of oocytes in the coelom (Table 1).

Fertilization *in vitro*

Table 2 presents the main characteristics of larval development of *Diopatra neapolitana* in this and in other studies (Bhaud & Cazaux 1987; Conti & Massa 1998).

Larval development was followed up to the age of 7 days. Seven hours after fertilization the embryo had cilia and swam in the water column, becoming a free-swimming protrochophore larva after 19 h. The protrochophore larvae were sub-spherical, with an apical tuft and were almost completely covered by cilia 210 μm in length. At 2–3 days after fertilization, the metatrocophore larvae had a length of between 240 and 280 μm and were segmented in three chaetigers with chaetae; the prostomium was ciliated and with two red eyes. After 3 days, the metatrocophore larvae lost the apical tuft, had four chaetigers and a length of 300 μm. On the 4th day, some metatrocophore larvae swam slowly in the water column, and others started to sink to the bottom and aggregate detritus around them. At this phase, larvae were moved to an aquarium with fine sediment and fresh sea water to allow the larvae to create a wrapping and protecting niche, and later permit the construction of the tube.

Juveniles were observed 7 days after fertilization and had five chaetigers, five small antennae on the prostomium, and a pair of small anal cirri in the pygidium. Tube formation was not observed, although individuals with particles around the body were seen.

Regenerating specimens

During the study period, about 5% of the specimens were regenerating the anterior end of the body, from two to 13 chaetigers. A minor proportion of specimens, about 0.3%, were regenerating the posterior end and a much larger number of chaetigers (56 to >100). Specimens regenerating the anterior end were found in almost all the sampling occasions, and represented between 1.4% and 17.0% of the sampled population. Individuals regenerating the posterior end were rare and only observed in 5 sampling occasions, randomly scattered throughout the sampling period. The majority of the regenerating specimens did not contain gametes, with the exception of some females with small oocytes.

Discussion

The study of the reproductive biology of *Diopatra neapolitana* showed that this species contained gametes in the coelom in all months of the year, but had the highest proportion of individuals with gametes from May to August. These results are similar to those of Dagli *et al.* (2005) in Izmir Bay, Turkey, where individuals with gametes were reported all year round, except in January. The number of oocytes in the females' body cavity was higher in May–August, but was decreasing during this period. In the month of October and December, it was similar numbers of oocytes were found in the body cavity

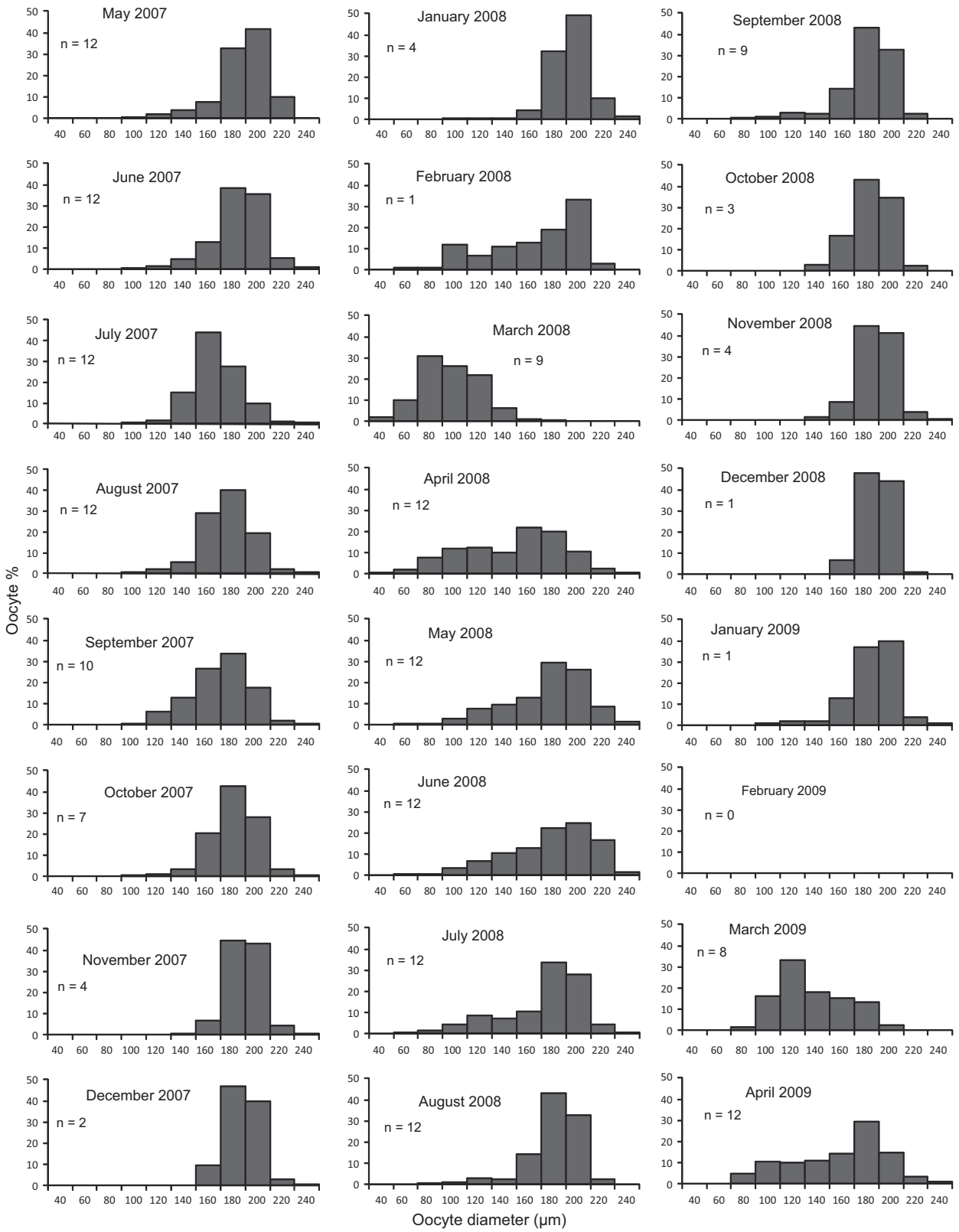

Fig. 5. Size-frequency distribution of oocytes of *Diopatra neapolitana* during the study period (n = number of females observed).

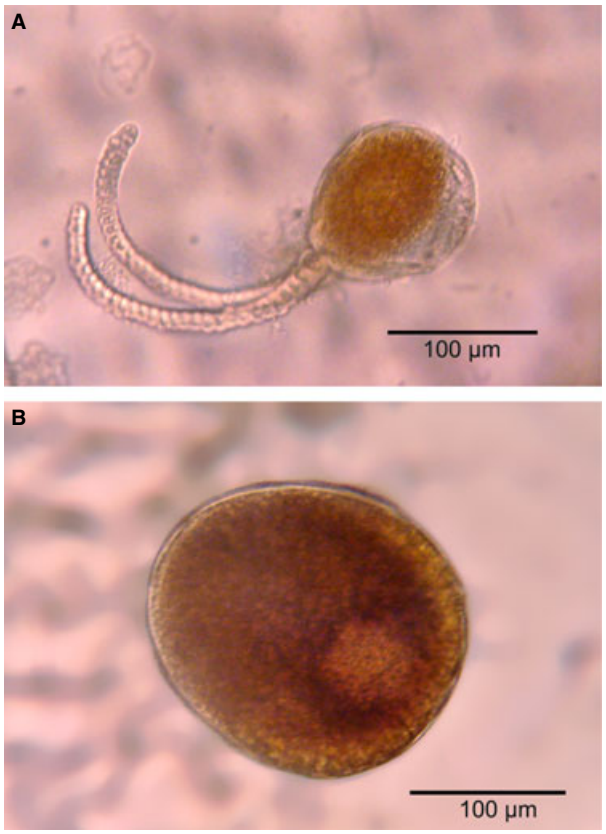

Fig. 6. Oocytes of *Diopatra neapolitana*. (A) Immature oocyte with nurse cells attached. (B) Mature oocyte.

of females, suggesting that no oocytes were released over this period. A number of small oocytes (<140 μm) was present almost every month, showing a peak in March and April, and then decreasing until September. The absence of small oocytes between November and January indicates that the females were not producing oocytes. The large oocytes probably were not expelled during spawning and remained in the coelom cavity. The decrease of small oocytes was paralleled by an increase in the number of larger oocytes.

Conti *et al.* (2005) described mature sperm of *D. neapolitana* as having a long tail attached to a spherical, short and rounded head, which is similar to our findings for sperm from May to August. The sperm also had the highest mobility during this period. These results indicate that the beginning of gametogenesis should be in March/April, the spawning period from May to August, and gametogenic inactivity from November to February.

The oocyte diameter varied between 40 and 240 μm, with a mean of 164.4 ± 40.8 μm. Oocytes should be released from the coelom into the water column with a diameter of about 200 μm (Dagli *et al.* 2005). In fact, in the present study only a small percentage of the oocytes remaining in the coelom had a larger diameter. This is in agreement with our observations of the artificial fertilization, and with Bhaud & Cazaux's (1987) results, as the fertilized eggs had a diameter between 210 and 215 μm.

Table 2. Principal characteristics of larval development of *Diopatra neapolitana* in this study and in comparison with Bhaud & Cazaux (1987) and Conti & Massa (1998).

	present study	Bhaud & Cazaux (1987)	Conti & Massa (1998)
P	19 h. Shape sub-spherical. Almost completely covered by cilia. Apical tuft. Larvae swimming actively in water column	24 h. Shape sub-spherical to piriform. Length 215 μm. Almost completely covered by cilia. Apical tuft. Larvae swimming in water column	5 h. Larvae swim free in the water column
M	2–3 days. Length 240–280 μm. 3 chaetigers. Prostomium ciliated. 2 red eyes	3 days. Length 240 μm. 3 chaetigers. Prostomium ciliated. 2 red eyes	24 h. Larvae present. Positive phototropism
M	3 days. Length 300 μm. 4 chaetigers. Loss of apical tuft. 2 red eyes. 4 days. 380 μm. 4 chaetigers. 2 red eyes. Some of them swimming in water column and others on the bottom, with detritus around them (starting the tube construction)	4 days. Length 390 μm. 4 chaetigers. Loss of apical tuft. 2 red eyes. 4–5 days. Larvae sink to the bottom and produce mucus where particles will aggregate	3 days. Larvae sink to the bottom. 4 days. Black jaws visible through body cavity. 3 chaetigers. Larvae start to agglutinate diverse detritus
E	5–6 days. 500 μm. 5 chaetigers, 2 red eyes. 5 large buds in prostomium. Rudimentary anal cirri. Black jaws visible through body cavity	6 days. Length 550 μm. 5 chaetigers. 5 large round antennal buds at the front of the prostomium. Black jaws visible through body cavity, rudimentary anal cirri	Not described
J	7 days. Length 540 μm. 5 chaetigers. 2 red eyes. 5 small antennae on the prostomium. 2 anal cirri. Juvenile present positive phototropism	7 days. Parapodia more developed. 5 antennae. 16 days. Length 1250 μm. 7 chaetigers. Parapodia and antennae more developed	1 month. 25 chaetigers. First branchia appears in 5th parapodia. 1 month and 20 days. Second branchia appears in 6th parapodia. 3 months. Length 15 mm

P, protrocophore; M, metatrocophore; E, erpochaeta; J, juvenile.

Nurse cells were observed in oocytes with a diameter equal or less to 160 μm, as reported by Dagli *et al.* (2005) and reached up to 39 cells, 230 μm long, and 12 μm wide, which is larger than observed by Dagli *et al.* (2005). Nurse cells are common in the Onuphidae family, and they probably transport nutrients taken up from the coelomic fluid to the developing oocytes. Usually, larger oocytes had few or no nurse cells attached, probably because nutrients will not be absorbed by the mature oocytes (Blake 1975).

During the study period, some individuals were regenerating the anterior end of the body. The regenerative capacity of the anterior segments has already been observed for *Diopatra* species, including *D. neapolitana* (Beli 2006). The majority of individuals that were regenerating the anterior end had no gametes in the coelom, except for some females that contained small oocytes. It is thus possible that the individuals in regeneration concentrate all their energy on this process.

The first chaetiger with gametes ranged in females from the 35th to the 70th and in males from the 50th to 70th, usually after the chaetigers with branchiae. These results are very similar to those obtained by Dagli *et al.* (2005), who reported the appearance of oocytes in chaetiger 55 ± 0.9 (mean) and of sperm in chaetiger 51 ± 0.9 (mean). In agreement with those authors, our study confirmed the absence of a significant correlation between the first appearance of the oocytes and the width of the 10th chaetiger, chosen as a measure of the total length of the specimens.

In the present study, the smallest mature male and female were 139.2 and 149.7 mm long, respectively. These values are higher than those obtained by Dagli *et al.* (2005), who reported minimum length in females of 125 and in males of 110 mm. However, in their study, the largest entire worm had a length of 347 mm, whereas in our study we found larger individuals. During our collecting period we harvested 46 complete individuals with a total length between 24 mm and 725 mm. Only specimens of about 600 mm long are reported in the literature.

According to Paxton (1993), there are four reproduction patterns in *Diopatra*: Group I – species that breed in the parental tube, Group II – species with direct development in a cocoon, Group III – species that attach their eggs to the parental tube and present direct development, and Group IV – species with broadcast spawning with a free-swimming larval stage. *Diopatra neapolitana* belongs to group IV, as no eggs were observed inside the tubes or any gelatinous mass containing eggs attached to their distal end. This conclusion is supported by the artificial fertilization experiment, where only free-swimming lecithotrophic larvae were observed. This is also in agreement with the conclusions of Dagli *et al.* (2005). In our fertilization experiment, free-swimming protrocophore larvae were obtained 19 h after fertilization, at 22 °C. In Conti & Massa (1998), this phase appeared 5 h after fertilization (25–32 °C) and Bhaud & Cazaux (1987) only observed the protrochophore larva 24 h after fertilization. The larvae developed faster in Conti & Massa's (1998) study compared with our study and Bhaud & Cazaux's (1987) description. Morphologically, the results of our experiment were similar to Bhaud & Cazaux's (1987) larval description, but the time of development was different, as in our study, larvae developed faster (about 1 day) until the metatrocophore phase.

These differences could be explained by the temperature, 22 °C in our case, and 25–32 °C in Conti & Massa (1998); temperature was never mentioned in Bhaud & Cazaux (1987). The number of larvae or the size of the containers could influence larval development, but we do not have information about these features. Larvae *of D. neapolitana* observed in our experiments and in Bhaud & Cazaux (1987) were morphologically different to those described by Choe (1960) in Japan as being *D. neapolitana*. This supports Paxton's (1993) suggestion that the species mentioned by Choe (1960) was not *D. neapolitana* (Paxton 1993).

Larval development has also been studied in other *Diopatra* species, namely *Diopatra cuprea* (Allen 1959), which has a developmental pattern similar to *D. neapolitana*, and *Diopatra marocensis* (Fadlaoui *et al.* 1995), which breeds in the parental tube. The first larval stage observed in this study in *D. neapolitana*, the protrochophore, was similar to that described for *D. cuprea* (Allen 1959) and *D. marocensis* (Fadlaoui *et al.* 1995). This stage is characterized in the three species by the presence of the apical tuft and ciliation around the body. *Diopatra neapolitana* and *D. cuprea* are active swimmers and had red eye spots during the initial development stages. *Diopatra cuprea* starts to settle to the bottom 3 days after fertilization, with four chaetigers, producing mucus to build the tube (Allen 1959). This was similar to what was observed in this study for *D. neapolitana*. Five antennae and anal cirri were observed at the 5th-chaetiger stage in *D. cuprea* and *D. neapolitana*, and at the 6th-chaetiger stage for *D. marocensis*. In *D. marocensis*, the ceratophores appear at the 12th-chaetiger stage and in *D. cuprea* at the 5th-chaetiger, with one to two rings, whereas in *D. neapolitana* the ceratophores still had no rings at the 50th-chaetiger stage (Conti & Massa 1998). According to these authors, the first branchiae appear at the 25th-chaetiger stage on the 5th chaetiger in *D. neapolitana*, and at the 18–20th chaetiger stage in *D. marocensis*, also on the 5th chaetiger (Fadlaoui *et al.* 1995).

In Ria de Aveiro, *D. neapolitana* is intensively exploited as live fish bait. No management or conservation regulations are currently set for the species and there is very

little legislation. In the case of the Sado estuary, located about 350 km south of Ria de Aveiro, harvesting of *D. neapolitana*, *Marphysa sanguinea* and *Hediste diversicolor* is not allowed from 1 November until 30 April (Portuguese legislation: Portaria no 576/2006 2006). That period is reported in the legislation as coinciding with spawning and juvenile growth. However, this is not supported by the present or other studies. The main reproductive period for *H. diversicolor* in the Sado estuary was from April to August/September (Garcês, unpublished data). In the Southwestern coast of Portugal (Odeceixe, Aljezur and Carrapateira) the same species was reported as reproducing throughout the year, with important peaks in September and May (Fidalgo e Costa 2003). In Ria de Aveiro, the species also showed two important reproductive periods, in March and September (Abrantes *et al.* 1999). The reproductive period of *Marphysa sanguine*a was mainly from March/April to October/November in the Sado estuary (Garcês, unpublished data) and a peak spawning period in April–May was reported from the Venice Lagoon (Italy) by Prevedelli *et al.* (2007). The main reproduction peak for *D. neapolitana* in the present study and in that of Dagli *et al.* (2005), in Izmir Bay (Eastern Mediterranean), was from May to August.

In Portugal, with the exception of the resting period established for the Sado estuary, the exploitation of polychaetes occurs all year, being more intense in warm months. Cancela da Fonseca & Fidalgo e Costa (2008) observed that the capture of these species has increased in recent years and that the mean size of harvested individuals is smaller. Dagli *et al.* (2005) reported that *D. neapolitana* occurred in the past in high densities in Inciralti (Mediterranean Sea), and by the time they did their study, the species was only present in their study area. They also observed that each digger needed 10 h to collect about 2000 specimens, whereas 10 years before they collected the same number in only 2 h.

The digging activity has negative impacts on the entire ecosystem. The benthic community is affected as a whole, as are the species which depend on it for food (mainly birds and fishes). In addition, the biogeochemical cycles could be affected and the release of nutrients and bio-availability of metals enhanced (Cancela da Fonseca & Fidalgo e Costa 2008). All of this emphasizes the urgent need for a sustainable exploitation of these natural resources, not only in Ria de Aveiro but in all coastal areas. The use of scientifically supported legislation coupled with control in the allocation of bait-digging licenses with regular monitoring of the impacted areas should be implemented. Mitigation measures could be applied either by restricting the harvest (in the case of *D. neapolitana* in Ria de Aveiro, the most suitable period seems to be April until September) or by establishing yearly rotating resting areas. The rotation system has been suggested as an effective solution to minimize the negative impacts of this kind of resource exploitation by Fowler (1999) and Cancela da Fonseca & Fidalgo e Costa (2008) and is being used in Korea, which is one of the largest exporters of bait polychaetes in the world (Choi 1985).

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