Biota Neotropica 20(3): e20200959, 2020
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)
Article

How oogenesis analysis combined with DNA barcode can help to elucidate taxonomic ambiguities: a polychaete study-based approach

***Bruno R. Sampieri*[1,2,3]*, *Tatiana M. Steiner*[4], *Priscila C. Baroni*[4], *Camila Fernanda da Silva*[4], *Marcos A. L. Teixeira*[2,3], *Pedro E. Vieira*[2,3], *Filipe O. Costa*[2,3] & *Antônia C. Z. Amaral*[1,4]**

[1]*Universidade Estadual de Campinas, Instituto de Biologia, Museu de Zoologia, Rua Charles Darwin, Bloco N, Cidade Universitária, Campinas, SP, Brasil.*
[2]*Universidade do Minho, Departamento de Biologia, Centro de Biologia Molecular e Ambiental, 4710-057 Braga, Portugal.*
[3]*Universidade do Minho, Instituto de Ciência e Inovação para a Bio-Sustentabilidade, Braga, Portugal.*
[4]*Universidade Estadual de Campinas, Departamento de Biologia Animal, Rua Monteiro Lobato, 255, Cidade Universitária, Campinas, SP, Brasil.*
**Corresponding author: Bruno R. Sampieri, e-mail: brunorsampieri@gmail.com*

SAMPIERI, B.R, STEINER, T.M., BARONI, P.C., SILVA, C.F, TEIXEIRA, M.A.L., VIEIRA, P.E., COSTA, F.O., AMARAL, A.C.Z. **How oogenesis analysis combined with DNA barcode can help to elucidate taxonomic ambiguities: a polychaete study-based approach**. Biota Neotropica 20(3): e20200959. https://doi.org/10.1590/1676-0611-BN-2020-0959.

Abstract: Polychaetes are common in coastal and estuarine environments worldwide and constitute one of the most complex groups of marine invertebrates. The morpho-physiology of the female reproductive system (FRS) can be understood by using histological tools to describe reproductive cycle and gametogenesis paths and, among other purposes, aiming to identify and differentiate polychaete species. However, this histology-based approach is rarely combined with molecular tools, which is known to accurately delimitate species. In the same way, the description and understanding of oogenesis and vitellogenesis paths within polychaetes are lacking for most families, narrowing the range of its utility. Therefore, the present study aims to describe the oogenesis in three polychaete species common and abundant on the South American Atlantic coast (*Laeonereis culveri, Scolelepis goodbodyi* and *Capitella biota*) and investigate the utility of reproductive features and gametogenesis as a relevant associate knowledge to discriminate species, particularly useful for putative cryptic species, integrated with morphological and molecular data. In a first attempt, the results obtained herein allow the authors to describe two new subtypes of oogenesis, dividing it in extraovarian oogenesis type I and II and intraovarian type I and II. The results also demonstrate that the following histological characters of the FRS can be relevant for the separation of related species: a) oogenesis type, b) occurrence or absence of a true ovary, c) ovary tissue organization, d) type of accessory cells present, and e) oocyte morphology. Additionally, these histological features of FRS, when compared with correlated species studied under this scope, converge with the genetic data. The analysis of cytochrome oxidase I (COI) barcode sequences differentiates between North and South American Atlantic populations of *L. culveri* (16.78% genetic distance), while in S. *goodbodyi* and *C. biota* it discriminates them from their congeneric species. These results highlight the importance of multi-tool approach and shows that both FRS histology and histo-physiology, and DNA barcoding can be used to identify and discriminate cryptic species, which is usually not possible when using morphological characters. Besides, these characters may also be useful in differentiating related species, and/or geographically distinct populations among polychaetes.

Keywords: *Integrative taxonomy; "Polychaeta"; oogenesis; histology; COI; cryptic species.*

Como análises de oogênese combinadas com DNA barcode podem elucidar ambiguidades taxonômicas: uma abordagem baseada em estudos com poliquetas

Resumo: Os poliquetas são comuns em ambientes costeiros e estuarinos em todo o mundo e constituem um dos grupos mais complexos de invertebrados marinhos. A morfo-fisiologia do sistema reprodutor feminino (FRS) pode ser compreendida por meio de ferramentas histológicas para identificar e diferenciar estes anelídeos. No entanto, essa abordagem histológica raramente é combinada com ferramentas moleculares, amplamente conhecidas por delimitar espécies congenéricas ou crípticas com maior precisão. Do mesmo modo, a descrição e o entendimento da oogênese e vitelogênese dentre os poliquetas, para a maioria das famílias, é ainda limitado. Portanto, o presente estudo tem como objetivo descrever a oogênese em três espécies de poliquetas comuns e abundantes na costa sul-americana (*Laeonereis culveri, Scolelepis goodbodyi e Capitella biota*) e investigar a utilidade das características reprodutivas e da gametogênese como um conhecimento associado relevante para discriminar espécies, particularmente útil para espécies crípticas putativas, integradas a dados morfológicos e moleculares. Os resultados aqui obtidos permitiram descrever dois novos subtipos de oogênese, dividindo-a em oogênese extra-ovariana dos tipos I e II e intra-ovariana dos tipos I e II. Os resultados também demonstram que os seguintes caracteres histológicos do FRS podem ser relevantes para a separação de espécies relacionadas: a) tipo de oogênese, b) presença ou ausência de um ovário verdadeiro, c) organização tissular ovariana, d) tipo de células acessórias presentes e, e) morfologia do ovócito. Além disso, essas características histológicas do FRS, quando comparadas às espécies correlatas estudadas sob esse escopo, convergem com os dados genéticos separando espécies putativas e congenéricas. As análises com DNA barcode demonstraram que em *L. culveri* é possível diferenciar as populações atlânticas Norte e Sul-americanas (16,78% de distância genética), enquanto para *S. goodbodyi* e *C. biota* fica evidente sua distinção com espécies congenéricas. Esses resultados destacam a importância da abordagem com múltiplas ferramentas e mostram que tanto a histologia quanto a histo-fisiologia do FRS e o DNA barcode podem ser usados para identificar e discriminar espécies crípticas e potencialmente crípticas, o que geralmente não é possível quando se utilizam apenas caracteres morfológicos. Além disso, esses caracteres também podem ser úteis na diferenciação de espécies relacionadas e / ou populações geograficamente distintas desses poliquetas.
Palavras-chave: *Taxonomia integrativa; "Polychaeta"; oogênese; histologia; COI; espécies crípticas.*

Introduction

Polychaetes reproduce mainly sexually, however asexual reproduction is commonly found within the group. Both forms present a great diversity of reproductive and developmental modes, in which the reproductive system's morphology itself, and oogenesis and vitellogenesis paths, display relevant characters for a broad range of research applications (Schroeder & Hermans 1975, Eckelbarger 2001, Rouse & Pleijel 2001, Aguado et al. 2014). Among individuals that reproduce sexually, the majority is dioecious and presents a very simplified reproductive system when compared to other invertebrates. In some cases, there are no tissues and / or organs specialized in the production of germ cells, in its storage or transportation to other body regions and even to the external environment. On the other hand, some species such as *Bathykurila guaymasensis* Pettibone 1989 and other deep-sea annelids show a complex and well tissue-organized reproductive system (Schroeder & Hermans 1975, Eckelbarger 2001, Rouse & Pleijel 2001, Glover et al. 2005, Aguado et al. 2014, Faroni-Perez & Zara 2014).

In polychaetes, oogenesis occurs in two distinct stages: the proliferative phase, in which the oogonia duplicates by mitosis; and the growth phase, in which oogonia I (pre-meiotic) and oogonia II (pre-vitellogenic) go through meiosis and initiate the maturation process (hypertrophy and vitellogenesis). In some species, the oogonia II exhibits cytoplasmic bridges between two or more cells at the end of the meiosis, while in others these bridges are observed in the early stages of growth phase (oocytes) (Adiyodi & Adiyodi 1983, Eckelbarger 2005). Among the studied species to date, two basic forms (types) of oogenesis were described: **a)** intraovarian, where oocytes develop completely within an ovary, associated or not with follicular cells; and **b)** extraovarian, in which the final differentiated oogonia or the immature oocytes detach from their proliferative tissue and reach the coelomic cavity, where it conclude development (Wilson 1991, Eckelbarger 1994, 2001, 2005).

The histo-physiological variations found in gametogenesis can provide important information on how these reproductive modes evolved among invertebrate taxa, especially in Annelida (Rouse & Pleijel 2001), which is of great value for evolutionary and taxonomic studies. In the same way, reproductive and gametogenesis features within polychaetes have been identified as useful for construction of phylogenetic hypothesis, such as vitellogenesis paths and/or oocyte morphology (Faroni-Perez & Zara 2014). Furthermore, studies regarding polychaetes gametogenesis have been done in only 0.1% of the described species (Eckelbarger 2005), and in the Americas these studies are restricted to few species that are ecologically relevant for environmental monitoring, usually pertaining the life-history or reproductive cycle (Eckelbarger 2005, MacCord & Amaral 2007, Garraffoni et al. 2014).

Faroni-Perez & Zara (2014) and Nunes et al. (2017) performed histochemical, ultrastructural and phylogeographic studies, in a complementary way, showing evidence of intraspecific variation in reproductive features on a presumed cosmopolitan species along the Atlantic waters, *Phragmatopoma lapidosa* Kinberg, 1866, and molecular evidence confirming the existence of two distinct species between North Western and South Western Atlantic regions.

Glover et al. (2005) and Meißner & Götting (2015) also used histological features to describe and delimitate annelid species; the first elucidate reproductive characteristics of *B. guaymasensis* common with congeneric species and with other hydrothermal vent annelids, while the second presents histological differences in tissue composition in the ventral epidermal glands of representative Spionidae from Australia. Meanwhile, other species, such as *Laeonereis culveri* (Webster 1879) (Nereididae), *Scolelepis goodbodyi* (Jones 1962) (Spionidae) and *Capitella biota* Silva & Amaral 2017 (in Silva et al. 2017) (Capitellidae), which are common and very abundant in tropical and subtropical Atlantic shallow waters and/or intertidal zones of South American coasts, have not been studied under this scope (Omena & Amaral 2001, MacCord & Amaral 2007, Oliveira 2009, Silva et al. 2017).

The use of molecular tools like DNA barcoding for specimen identification and classification has been shown to be successful in several marine groups (Radulovici et al. 2009, Knebelsberger et al. 2015, Raupach et al. 2015). Its usage has become quite widespread in marine invertebrates, often as a complement to morphological identifications and providing a quick screening method for highlighting mismatching morphological and molecular data, and detect putative cryptic species, species complexes, and inaccurate or misleading identifications (Hebert et al. 2003, Hajibabaei et al. 2006, Costa & Antunes 2012, Lobo et al. 2016). In this way, integrative approaches encompassing one or more morphological analyses (i.e. histology, transmission electron microscopy, scanning electron microscopy) with molecular data, namely DNA barcodes, are desirable and encouraged for a better taxonomic resolution (Langeneck et al. 2020; Martin et al. 2020; Teixeira et al. 2020)

Given this scenario, this study aims to describe female gametogenesis in these three species of polychaetes, bringing to light new data regarding oogenesis of this group and investigate the utility of histology as a relevant associate tool to discriminate species, particularly useful for putative cryptic species, which are supposed to be distinguishable through molecular methods only. Considering the apparently high incidence of cryptic species among polychaetes (Nygren 2014, Lobo et al. 2016), we anticipate that the application of histological and histo-physiological analysis will be particularly relevant for the taxonomy and systematics of this highly diverse group of invertebrates.

Material and Methods

1. Collection of specimens

The following species were analyzed: *Laeonereis culveri* (20 specimens), *Scolelepis goodbodyi* (20 specimens), both collected in Araçá Bay, São Sebastião, Brazil (23º48'49.9"S 45º24'31.3"W), during the summer months of 2016; and *Capitella biota* (five specimens), collected at Praia do Perequê, Guarujá, Brazil (23º56'31.0''S 46º10'25.3''W), in the early fall of 2017. The analyzed material was collected manually with a shovel in days of low syzygy tides. These species were studied for four reasons: **1)** they are abundant in sandy and muddy bottoms of the intertidal region; **2)** belong to distinct families; **3)** in the case of *L. culveri*, taxonomic or identification ambiguities/issues are reported; and **4)** reproductive cycles of congeneric species are documented in the literature, hence allowing comparisons.

2. Histology

For the histological analysis, at least five ovigerous females of each species were chosen by the observation of oocytes in their coelomic cavity and fixed in 10% glutaraldehyde solution in Phosphate Saline Buffer (PBS) with addition of 7% sucrose. After a minimum of 72 h below 4 ºC, the subjects were washed in PBS for 5 min and photographed using a stereomicroscope (Zeiss Axio Zoom Imager M2). Some of the ovigerous females were dissected to release the oocytes from the body cavity to describe their external morphology.

The same individuals were then dehydrated in 70, 80, 90 and 95% ethanol for 15 min at each concentration. Following the dehydration, infiltration with embedding historesin was performed for at least seven days, and then the final inclusion in Leica historesin for blocks polymerization was conducted. Each block was sectioned in microtome (Leica RM2245) in slices of 3.5 µm each, collected on glass slides and stained with Harris - eosin hematoxylin for further photo documentation under light microscopy (Zeiss Axio Imager M2). A total of 18 slides were analyzed for each species.

3. DNA extraction and amplification

Five *L. culveri* and six *S. goodbodyi* specimens both sampled from the Araçá Bay were fixed in ethanol 99% and used in subsequent molecular analysis. The only exception was *C. biota*, because cytochrome oxidase I (COI) barcode sequences from this species were already available from the same sampling location (Silva et al. 2017) and the specimens were confirmed by the authors.

DNA extraction was performed using the E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek) according to manufacturer instructions. A small amount of tissue of each specimen was used. Then, the 658-base pair (bp) fragment from the 5'end of COI was amplified using the set of primers PolyLCO/PolyHCO (Carr et al. 2011). All PCR reactions were performed in a 25 µl volume containing 2.5 µl of 10X PCR buffer + KCl, 2.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTPs, 0.2 µl of Taq polymerase (Thermo Fischer Scientific) and 1.5 µl of each primer (10 mM). DNA template varied between 2 µl and 4 µl. Cycling conditions for PCR reactions with the primer pair PolyLCO/PolyHCO were: one cycle of 94 ºC for 1 min, 5 cycles of 94 ºC for 40 s, 45 ºC for 40 s and 72 ºC for 60 s, 35 cycles of 94 ºC for 40 s, 51 ºC for 40 s and 72 ºC for 60 s, with a final extension of 72 ºC for 5 min. Amplification success was checked in a 1.5% agarose gel, using 5 µl of PCR product, and successful PCR products were then purified (ExoSAP protocol - Thermo Fisher Scientific). Cleaned-up amplicons were sent to external sequencing service suppliers (Macrogen Europe, Spain), for bidirectional sequencing.

The sequences obtained for *L. culveri* and *S. goodbodyi* in the present study were deposited in BOLD under the dataset "SCLAE" DOI: dx.doi.org/10.5883/DS-SCLAE. All sequences are also available at the GenBank and accession number is provided for each sequence mined from database in phylogenetic trees presented herein.

4. Genetic analysis and data treatment

All sequences were analyzed and edited using MEGA 7.0 (Kumar et al. 2016). Trace files were checked manually, unreadable zones and primers removed, and ambiguous bases corrected.

Then, the edited sequences were aligned using Clustal W (Thompson et al. 1994) implemented in MEGA 7.0 (Kumar et al. 2016) and the translation verified for stop codons or indels. GenBank BLASTn search (Altschul et al. 1990) and BOLD Identification System tool (Ratnasingham & Hebert 2007) were used to search for similarity to confirm the target taxa.

When publicly available (in GenBank and/or BOLD), representative COI sequences of the same or congeneric species were added to the genetic analysis. Sequences publicly available that raised uncertainty regarding their confidence were excluded. To assure this proofreading, two steps were applied. First, all available sequences were pre-screened for codon-stops, indels, sequence length (more than 500 bp) and ambiguous or incomplete taxa names. Then, a second step was done to evaluate misidentifications by constructing a preliminary neighbor-joining tree in MEGA 7.0 (Kumar et al. 2016) using Kimura-2-parameter model (1 x 10^3 bootstraps of support). Whenever distinct taxa clustered together, the more represented taxa or the ones associated to a peer-reviewed publication were accepted.

Interspecific (or between geographic distant populations) distances were calculated using pairwise distances (1000 bootstraps replicates) in MEGA 7.0 (Kumar et al. 2016). Maximum likelihood (ML) trees for COI were constructed based on the best-fitting model of nucleotide substitution implemented in MEGA 7.0 (Kumar et al. 2016) for each group: GTR+G+I (for *L. culveri* and *S. goodbodyi)* and HKY+G (for *C. biota*).

Results

1. *Morphology of reproductive characters*

Table 1 shows a summary of the FRS morphological and histological features obtained through the comparative oogenesis analysis of the studied polychaete species.

1.1. Laeonereis culveri (Figure 1A-C)

In this species, it is possible to observe oocytes through the specimen tegument floating in the coelom (Figure 1B). They are large, spherical cells and occur in varying numbers according to the female's reproductive stage. In *L. culveri* females that are in an early stage of gametogenesis, primordial germ cell clusters can be observed in some setigers, presented as a white spherical "spot" when observed with naked eye. Under stereomicroscope, this cluster is most clearly seen with a lobed shape (Figure 1C).

1.2. Scolelepis goodbodyi (Figure 1D-F)

In *S. goodbodyi* a different organization and distribution of germ cells and their original tissues can be observed. No free-floating oocytes were observed in the specimen's coelomic cavity, but oval yellowish oocytes packaged in the parapodia of each setiger after the 24th or 25th segment (Figure 1E). Inside the germinal setiger a sac-like tissue is observed, a sheath that houses the developing germ cells, trapped in the specimen coelomic wall (Figure 1E). The oocytes in advanced stage of vitellogenesis are elliptic cells with an oval and centralized germinal vesicle (Figure 1F). The oocytes surface is very rich in membrane specializations (villi) which form a honeycomb-like net, just below which a thin darker line is observed suggesting that the deposition of the shell begins at the end of the vitellogenesis and just below these villi (Figure 1F).

1.3. Capitella biota (Figure 1G-I)

In this species, some organization was also observed in the distribution of oocytes in the coelom, in which the occurrence of oocytes from the fifth setiger of the specimen can be noticed, arranged in pairs in each segment and not more than six in number. They are large, elliptic cells with round germinal vesicles (mature oocytes) (Figure 1H and I), showing some disproportionality regarding the dimensions of the adult animal. The coelom is full of follicular cells that appear in clusters or individually (Figure 1H).

2. *Histology*

The following germ cells were observed at different developmental stages: **a)** primary oocytes (**poo**), cells in the pre-meiotic stage (interphase) or onset of meiosis II; **b)** secondary oocytes (**soo**), end-stage meiosis II cells; and **c)** mature oocytes (**Oo**) showing different morphological features due to vitellogenesis process.

Table 1. List of relevant histological characters of the female reproductive system for each species studied. (1) = species studied in this work; (2) = data obtained from Klesch (1970); (3) = data obtained from Richards (1970); (4) = data obtained from Eckelbarger & Grassle (1982, 1983). (*) Differences between species observed in the present study.

	SPECIES					
CHARACTERS	***L. culveri*** (1)	***L. culveri*** (2)	***S. goodbodyi*** (1)	***S. squamata*** (3)	***C. biota*** (1)	***C. teleta / C. jonesi*** (4)
Oogenesis type	Extraovarian type II (*)	Extraovarian type I (*)	Intraovarian type II (*)	Intraovarian type I (*)	Intraovarian type II	Intraovarian type II
Ovary organization and location	Lack	Lack	Paired organ in each setiger	Paired organ in each setiger	Paired organ in each setiger; follicles; isolated oocytes (*)	Paired organ in each setiger; follicles; (*)
Type of acessory cell	Follicular cells (*)	Lack (*)	Follicular cells	Follicular cells	Follicular cells	Follicular cells
Possible source of pre-vitelinic substances	Coelom and follicular cells (*)	Coelom and parenchymal tissue (*)	Blood vessel, gut, follicular cells (*)	Follicular cells and coelom (*)	Gut, follicular cells and close oocytes	Gut, follicular cells and close oocytes

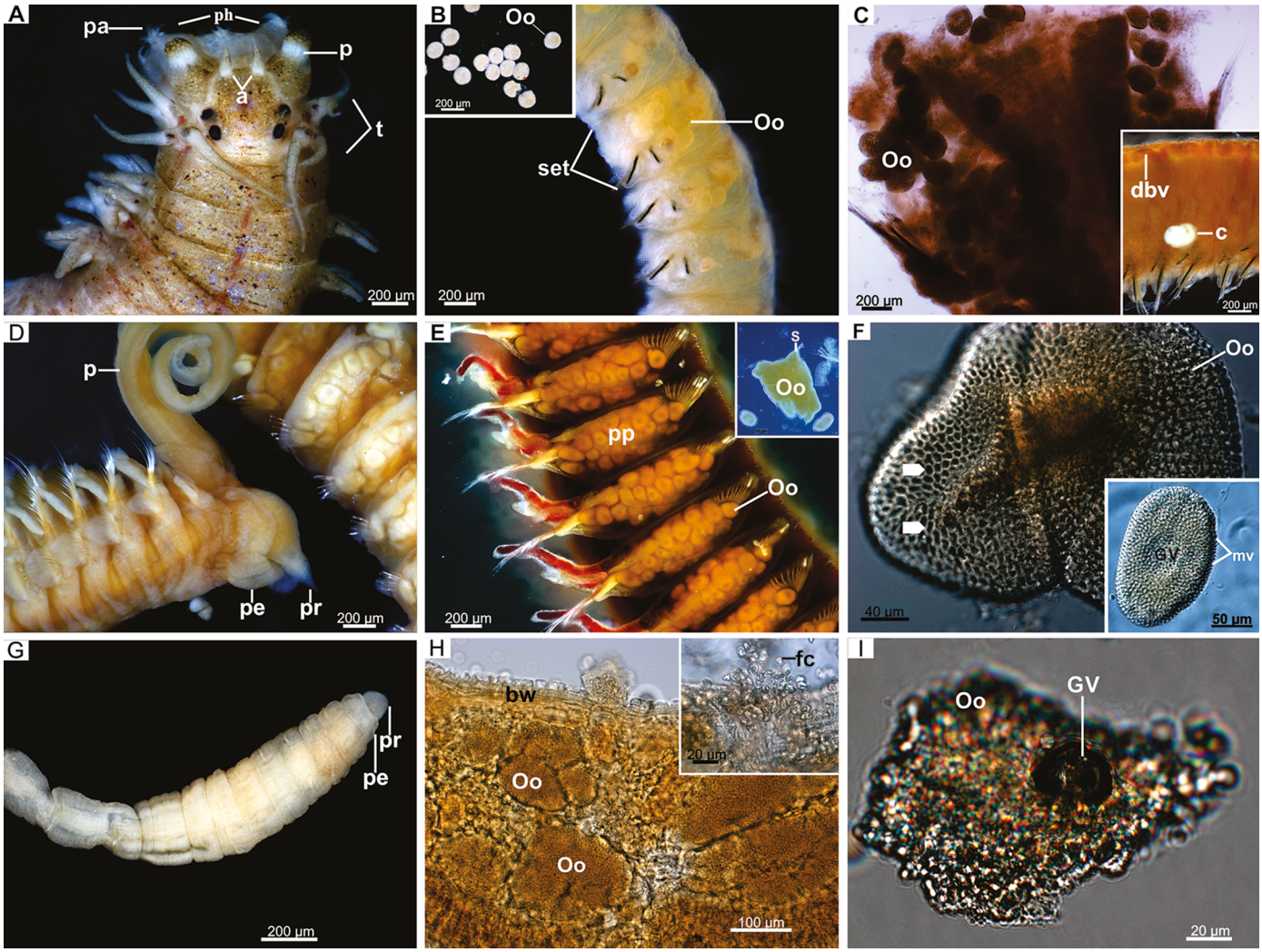

Figure 1. *Laeonereis culveri*, *Scolelepis goodbodyi* and *Capitella biota* external morphology, highlighting the germ cells and its macro organization. **A – C:** *Laeonereis culveri*; **A – Anterior body dorsal** view; **B –** Germ setigers; detail: mature oocytes visible through the tegument; **C –** Release of free oocytes from the coelom by rupture of the body wall; detail: primary and secondary oocytes cluster seen through the female tegument; **D – F:** *S. goodbodyi*: **D –** Anterior body and germ setigers; **E –** Germ setigers in lateral view, showing parapodia full of oocytes; detail: epthelium sheath (sac-like tissue) holding oocytes; **F –** External morphology of oocytes evidencing the organization of honeycomb-like membrane projections; detail: macrovilli; **G – I:** *C. biota*; **G –**Anterior body, dorsal view; **H –** Distribution of follicles in the germ setigers observed through the integument; detail: free follicular cells after body wall disruption; **I –** Detail of a free oocyte released from the follicle. **a** = antennae; **arrowhead** = honeycomb-like structures; **bw** = body wall; **c** = cluster; **dbv** = dorsal blood vessel; **fc** = follicular cell; **GV** = germ vesicle; **mv** = macrovilli; **Oo** = oocyte; **p** = palps; **pa** = pharynx papilae; **pe** = peristomium; **ph** = pharynx**;** **pp** = parapodia; **pr** = prostomium; **set** = setiger; **t** = tentacles.

2.1. Laeonereis culveri

In *L. culveri* no differentiated reproductive system was properly observed and clusters of oocytes in different development stages are free-floating in the coelom (Figure 2A-C). Primary oocytes clusters are free of follicular cell sheath (Figure 2B), which seems to start its connection to germ cells only after the latter become secondary oocytes and initiate vitellogenesis. Primary and secondary oocytes are small and rounded cells whose cytoplasm is homogeneous, free of apparent yolk vesicles and with restricted space due to the size of the nucleus. The nucleus, in turn, is large, also rounded, with condensed peripheral chromatin, occupying most of the intracellular space (Figure 2B, C and E).

Somatic cells that are associated with oocytes are follicular cells that show a heterogeneous cytoplasm and chromatin, suggesting a high synthesis activity, mainly when they are related to oocytes in advanced vitellogenesis (Figure 2D and F). The oocytes observed in this species seems to undergo non-synchronous vitellogenesis, due to distinct characteristics between oocytes inside a single cluster. Oocytes initiating vitellogenesis are characterized by rounded cells, with homogeneous cytoplasm still without yolk vesicles in which the nucleus occupies the center of the cell and presents a loose chromatin, indicating synthesis activity. In other oocytes, morphologically similar to previous ones, few yolk vesicles accumulating at the periphery of the cell are observed. Larger oocytes with a greater yolk accumulation can be seen in the same cluster (Figure 2D-F).

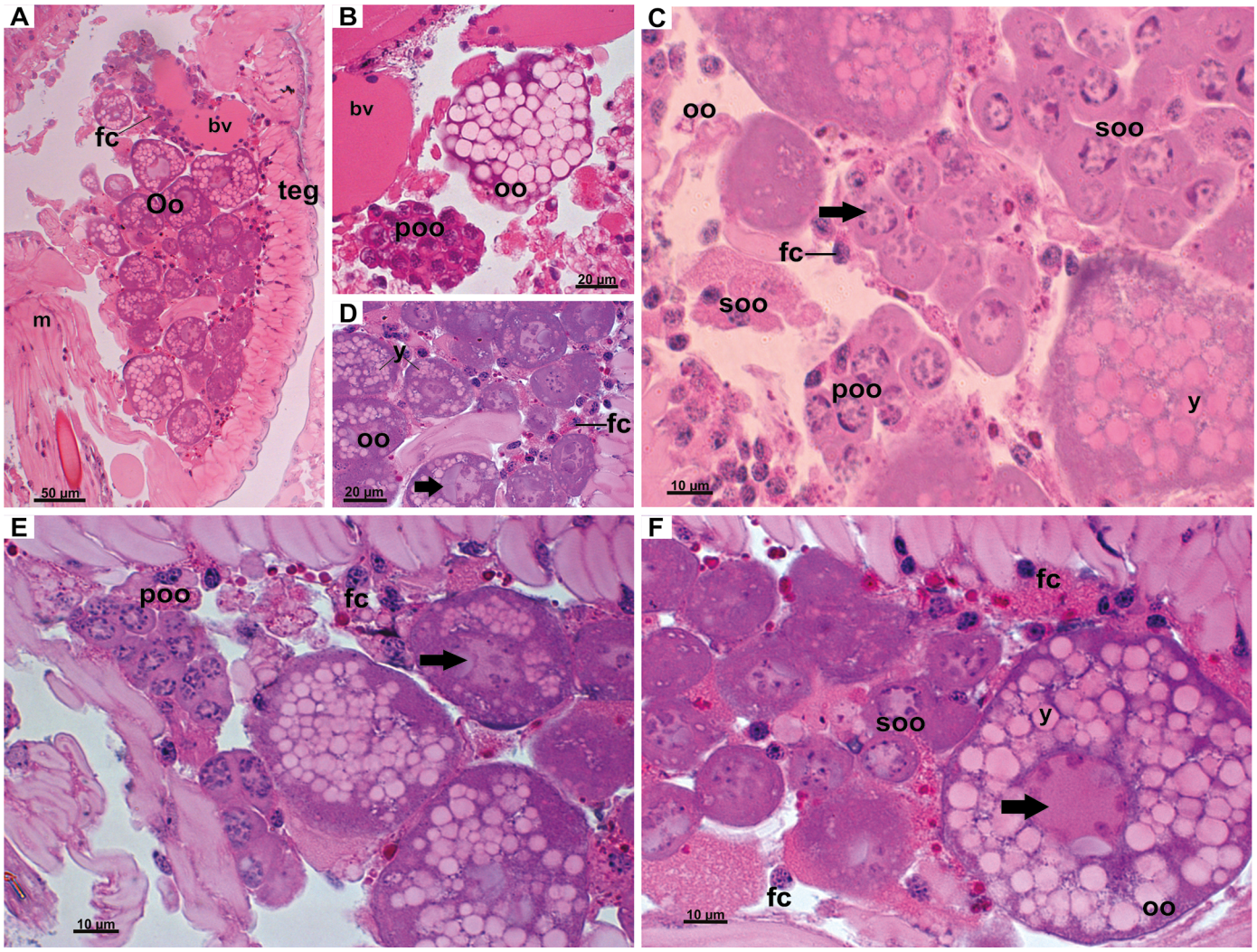

Figure 2. *Laeonereis culveri* histological sections with emphasis on its germ cells and vitellogenesis. **A – D:** Histological sections of parapodia where primary and secondary oocytes clusters are observed, associated to blood vessels and follicular cells (soo); **E – F:** Detail of primary and secondary oocytes clusters and oocytes in vitellogenesis. **Arrow** = germ vesicle (nucleus); **bv** = blood vessel; **fc** = follicular cell; **m** = muscle; **Oo** = oocyte; **poo** = primary oocytes; **soo** = secondary oocytes; **teg** = tegument; **y** = yolk.

In oocytes in which the vitellogenesis is more advanced, the yolk vesicles are becoming larger in a fusion process and in some cases, it is not possible to observe the germinal vesicle (nucleus). In such cases, yolk vesicles occupy the whole of the cytoplasm and the characteristics become striking, even altering the rounded form of the oocyte, and the shell begins to be deposited in the external cellular limit (Figure 2A-C). Furthermore, these oocytes are still observed associated with follicular cells (Figure 2D, E and F).

2.2. Scolelepis goodbodyi

In this species, it is possible to observe an epithelial sheath (peritoneum) that compacts and shelters the oocytes isolating them from the other organs immersed in the female coelomic fluid, restricted to the parapodia (Figure 3A-B). The most immature germ cell stage found here is secondary oocytes, arranged in pairs, without cytoplasmic bridges (as far as can be seen with this technique) and associated to a blood vessel by one of their poles. They are oval/elliptic cells with round nuclei, cytoplasm slightly granular (heterogeneous) but with no evidence of yolk vesicles (Figure 3A).

In this species, oocytes initiating vitellogenesis are cells that have undergone a marked hypertrophy, exhibiting double or more of secondary oocytes' size. They are cells elliptically shaped, whose cytoplasm exhibits signs of yolk granulation. The germinal vesicle retains its round shape, but with little condensed chromatin and a large nucleolus strongly stained by hematoxylin (Figure 3A-B). Continuing the vitellogenesis process, the cells almost double in size and the cytoplasm is already found with a thin yolk granulation easily observed. The formation of membrane specializations, or projections, at the border of the cell create intimate contact with other oocytes, follicular cells and with blood vessels. In the next developmental step, a greater amount of yolk vesicles within the cytoplasm and larger and more developed membrane projections can be observed as oocytes main features (Figure 3B-D).

Among oocytes in final stages of vitellogenesis, a considerable increase in the yolk assimilation and synthesis is observed due to the larger quantity of vesicles, also of larger size, occupying the cytoplasm almost completely, besides a well-developed germinative vesicle with loose chromatin and nucleolus well evident.

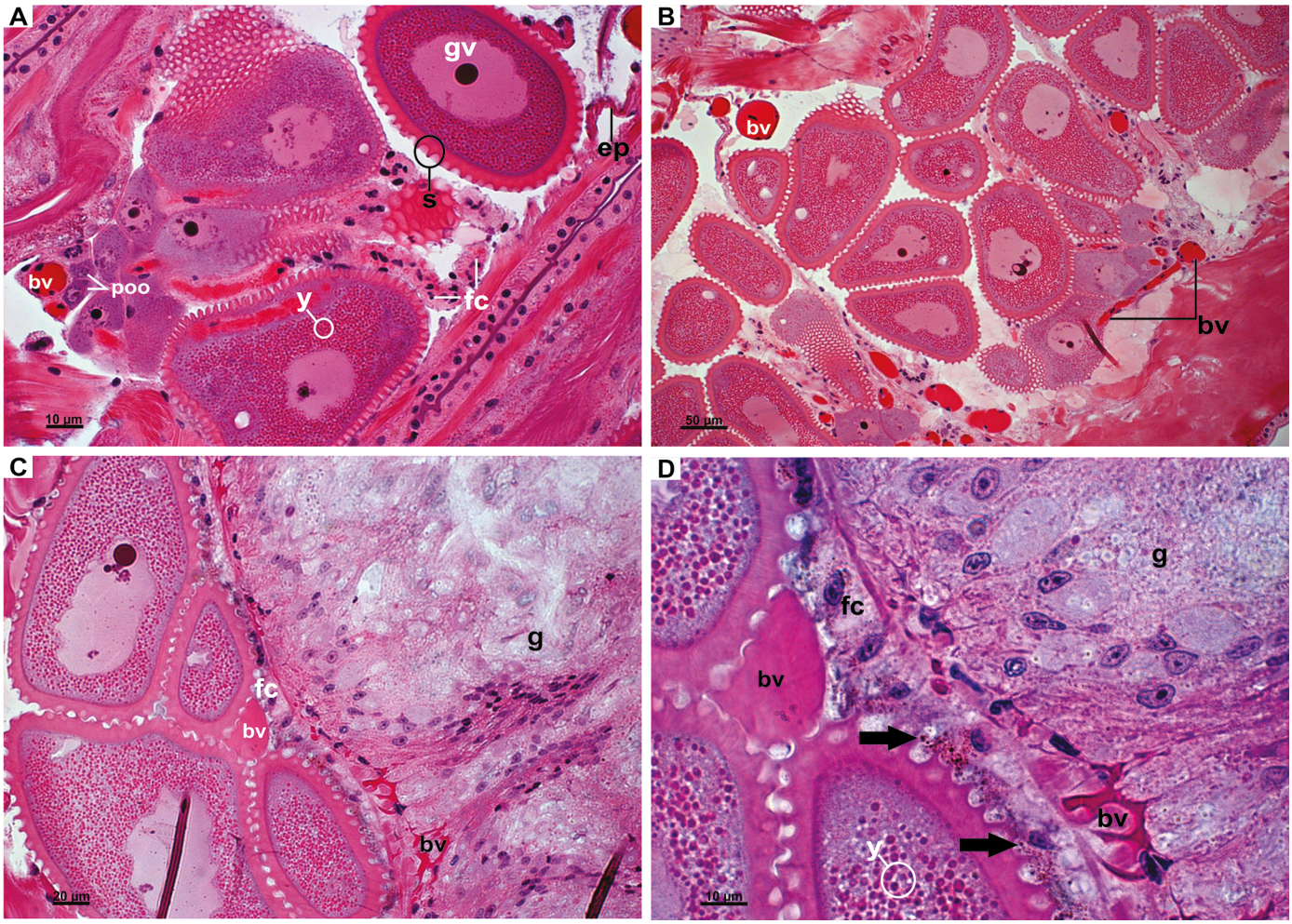

Figure 3. *Scolelepis goodbodyi* histological sections with emphasis on its reproductive system, germ cells and vitellogenesis process. **A:** Parapodia section showing an ovary filled with germ cells from the initial to the advanced stages of development; **B:** Details of blood vessel close to primary oocytes and vitellogenic oocytes; **C:** Detail of blood vessel very close to vitellogenic oocytes and follicular cells, which are in contact with gut wall; **D:** Magnification of previous image, showing a possible material transfer between the gut, blood vessel and follicular cells to oocytes (**arrow**). **bv** = blood vessel; **ep** = epithelium; **fc** = follicular cell; **g** = gut; **gv** = germ vesicle; **poo** = primary oocytes; **s** = shell; **y** = yolk.

At this stage, the shell deposition between the membrane projections is observed, covering the entire layer of villi on the cell surface when complete and tending to lose contact with other cells, including follicle ones (Figure 3A, 3C-D).

The follicular cells found in this species seem to play a role in vitellogenesis, evidenced in the figures 3B and 3D, not only by direct contact with the oocytes, but also by association with blood vessels and by the external wall of the individual's gut. Germ cell support within the epithelial sheath appears to be a function of the follicular cells associated with blind capillaries, where they form peduncles similar to grape clusters, maintaining an interconnected network between follicular cells, gut, blood vessels and oocytes.

2.3. Capitella biota

This species, as others congeneric ones, presents a rudimentary ovary, with a paired and bead necklace organization and its walls are formed by an epithelium anchored in the dorsal peritoneum in its upper portion and longitudinally in the septa of each segment crossing the entire set. The epithelium that connects the organ to the inner face of the body wall and to the septa has cubic cells, while the portion involving each oocyte individually (forming the follicles), exhibits a single layer of pavemented cells (Figure 4A and B).

As previously stated, *C. biota* oocytes are very large cells (250 µm in length) when compared to the body size of the specimen, but in early stages of vitellogenesis, oocytes are tiny cells with few yolk vesicles (which increase in quantity and volume according to development) and a germinal vesicle prominent in the center of the cell (Figure 4A detail). As oocytes progress in vitellogenesis, the cell increases considerably in size and the yolk vesicles also increase in size and quantity while the germinal vesicle is almost not observed (Figure 4B). The largest and most developed germ cells observed here exhibit a rather increased, elongated and elliptical size, taking up half the full length of a setiger of the specimen, with the cytoplasm full of yolk vesicles covering the germinal vesicle (Figure 4C and D).

No cytoplasmic bridges were observed between the oocytes at any stage, but it was possible to notice an intimate contact of the oocytes with the many follicle cells found in the coelom, in addition to a proximity to the gut.

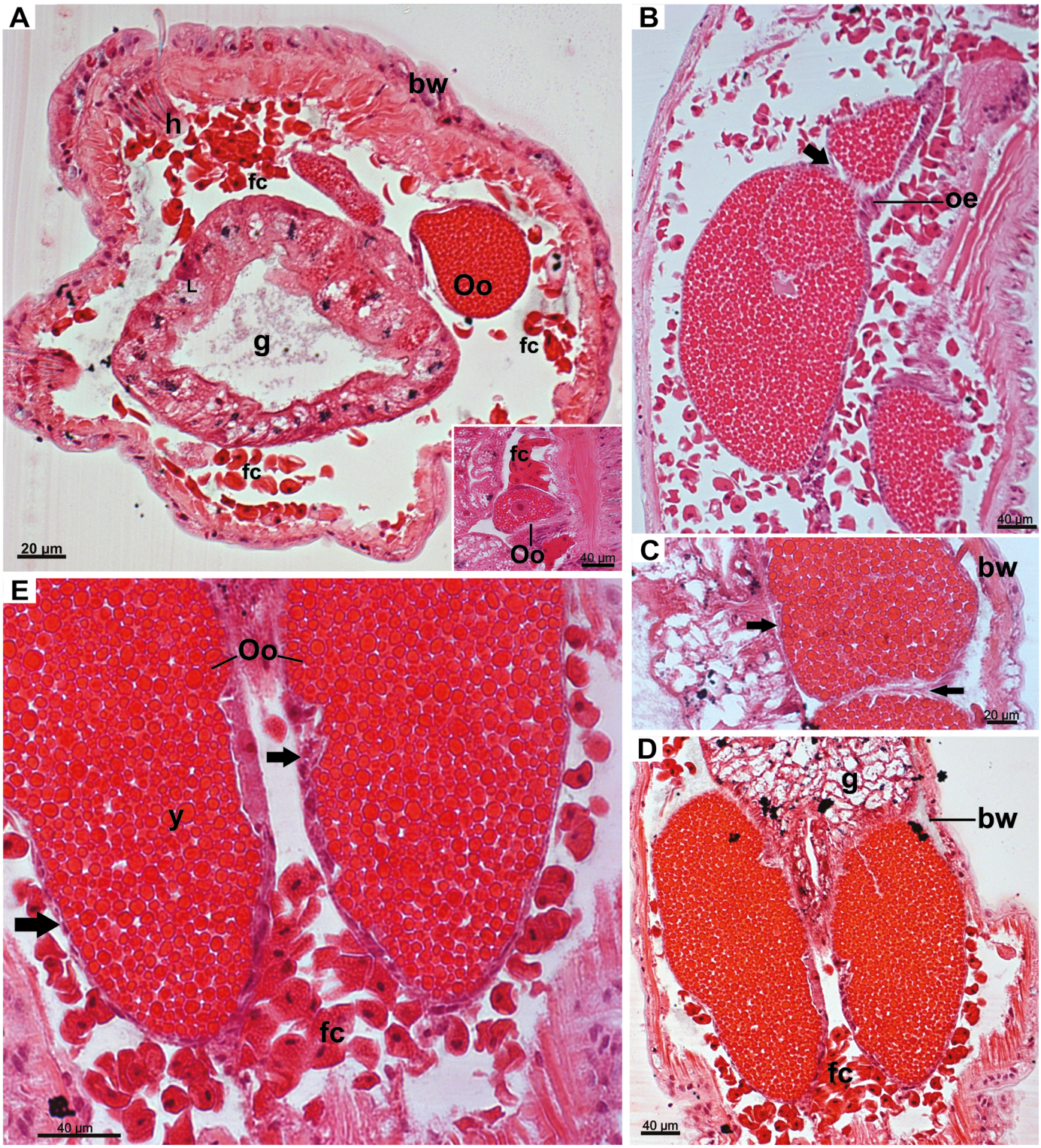

Figure 4. *Capitella biota* histological sections with emphasis on its reproductive system, germ cells and vitellogenesis process. **A:** Transverse section of the germ setiger, where a gut with dilated lumen is observed, surround by several follicular cells and a follicle with an oocyte in vitellogenesis process. Detail: Imature oocyte; **B:** An mature oocyte and the epithelial tissue that involves each germ cell; **C - E:** Details of the ovary epithelium housing each of the oocytes, surrounded externally by follicular cells. **bw** = body wall; **fc =** follicular cells; **g** = gut; **h** = hook; **oe** = ovary epithelium; **Oo** = oocyte; **arrow** = individualized oocytes; **y** = yolk.

These follicular cells are amoeboid-shaped, showing cytoplasm strongly stained by eosin and very heterogeneous, with a small and condensed nucleus, found in clusters associated with the ovary itself and also with the gut (Figure 4A-D).

3. *Molecular analysis*

For the three species studied here, the ML trees clearly discriminated them from geographic distinct populations (*Laeonereis culveri* – Figure 5A) or from congeneric species (*Scolelepis goodbodyi* – Figure 5B and *Capitella biota* – Figure 5C). Mean pairwise COI distances between *S. goodbodyi* and the other congeneric species ranged between 15 and 23%, between *C. biota* and the other congeneric species ranged between 18 and 21%, while for *L. culveri*, the pairwise distance between the populations from Brazil and North America was 16.78%.

Discussion

When comparing the species in this study regarding the shape and size of their oocytes, there are marked variations that reflect relevant ecological and/or reproductive aspects. In *L. culveri* the largest oocytes reach little more than 150 µm in diameter at the end of vitellogenesis and have a rounded/spherical shape, while the oocytes of *S. goodbodyi* and *C. biota* are bigger than 200 µm in length and have an elliptical shape when packed in the ovaries; also, in *C. biota*, the oocytes present a less definite shape when outside the ovary. These characteristics, based on Adiyodi & Adiyodi (1983), separate *L. culveri* from the other two species (as expected) due to the presence of large numbers of oocytes of smaller sizes, classifying it as a species of discrete iteroparity, whereas *S. goodbodyi* and *C. biota* show a different reproductive strategy, i.e., semi-continuous reproduction, in which individuals produce a smaller number of eggs, but with a larger size. *C. biota* has the smallest number of oocytes compared to the other two species, with ovarian follicles and individualized oocytes. Thus, with respect to the morphology and development of the female reproductive system, these three species are very distinct and well defined in their respective families.

Considering the studies carried out by several authors, including the present one, it is believed that the oogenesis in polychaetes occurs in two ways (Eckelbarger 2005), namely extraovarian (**a**) and intraovarian (**b**): **a)** final differentiated oogonia or primary oocytes detach from their proliferative tissue and reach the coelomic cavity, where they conclude development; and **b)** in which oocytes develop completely within an ovary, usually associated with follicular cells (Wilson 1991, Eckelbarger 1994, 2001, 2005) . In this study, the authors propose a subdivision of the two types described by Eckelbarger (2005), both of which display two subtypes:

a) extraovarian type I – the oocyte is released from the proliferative tissue as primary oocytes (pre-vitellogenic), individualized (solitary) within female's coelomic cavity, where full development occurs.

b) extraovarian type II – clusters of primary (pre-vitellogenic) oocytes, surrounded by a follicular cell sheath and/or a binder matrix, are released into the coelomic cavity for the entire process to occur.

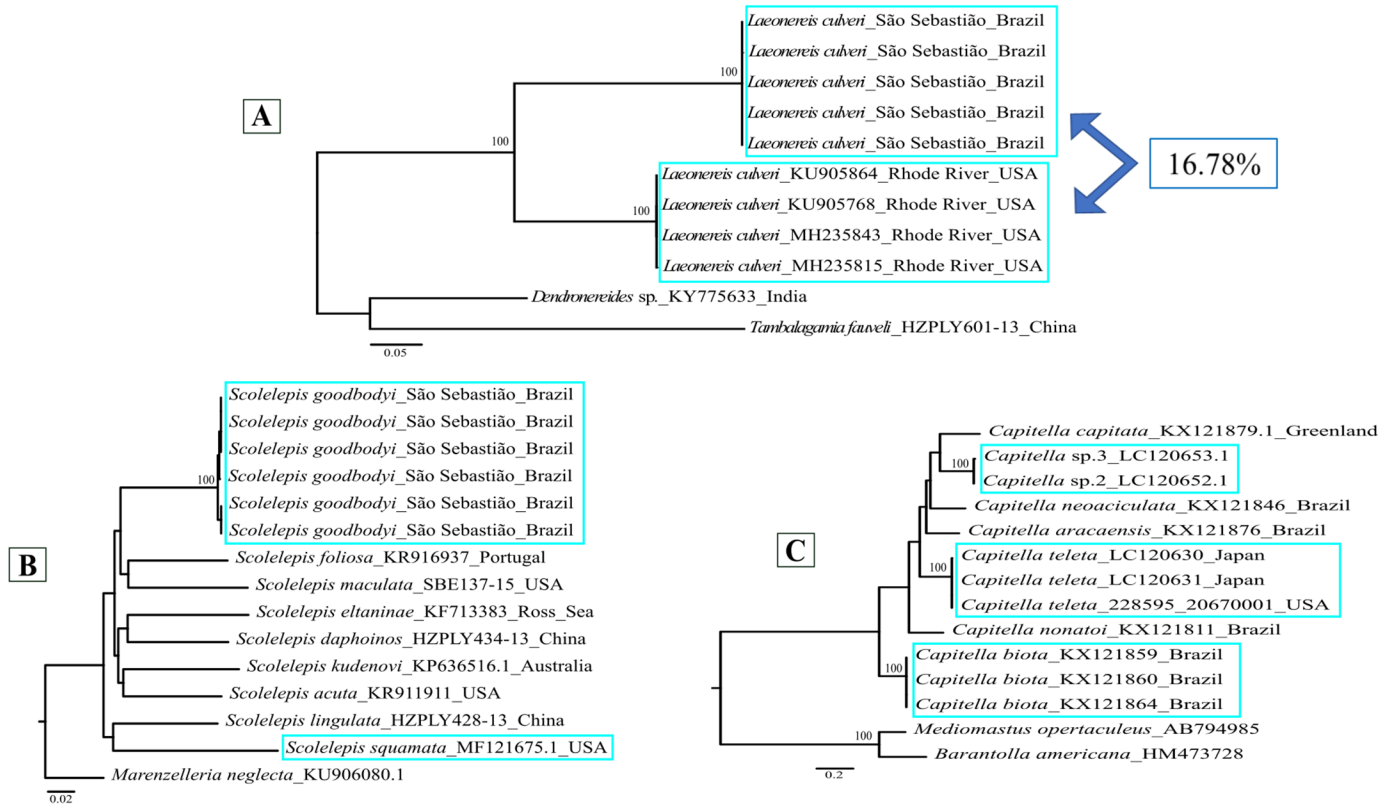

Figure 5. Phylogenetic trees of the species herein studied. Numbers by the nodes indicate respective maximum likelihood bootstrap values; values below 90 not shown. **A:** ML phylogenetic COI tree of *Laeonereis culveri*. The species *Dendronereides* sp. and *Tambalagamia fauveli* were used as outgroup. **B:** ML phylogenetic COI tree of *Scolelepsis goodbodyi*. The species *Marenzelleria neglecta* was used as outgroup. **C:** ML phylogenetic COI tree of *Capitella biota*. The species *Mediomastus opertaculeus and Barantolla americana* were used as outgroup. The COI sequence for *Capitella teleta* from USA is deposited under ID 228595, on the Genome Portal of the Department of Energy Joint Genome Institute (ESC-2004).

c) intraovarian type I – oogonias housed inside an epithelial sheath/sack proliferate and primary oocytes develop until the late phase of vitellogenesis, released into the coelomic cavity afterwards, ending this process and subsequent oviposition/fertilization.

d) intraovarian type II – oocytes packaged in epithelial sheath/sack, fully develop in their interior until the ovulation period.

This complementary data regarding oogenesis paths in polychaetes brings to light additional characters that can be used to differentiate other taxonomic levels, such as genus and species. Each species-case treated herein is discussed below, encompassing this and others characters observed, aiming to show how the FRS histology could be used as a tool to discriminate species.

1. The Laeonereis culveri *case: cryptic or cosmopolitan?*

In *L. culveri* from São Sebastião, Brazil, there is no true ovary. In this species, the germinative tissue (not observed herein) is distributed in pairs in the setigers and associated to blood vessels, and its observation depends on the technique used and life cycle stage (Eckelbarger 2005). The germ line cells originate from this tissue and the follicular ones from the peritoneum. In this way, ovulation in *L. culveri* occurs prior to the onset of vitellogenesis, consisting of a large number of primary oocytes being produced and released in clusters within the adult coelom, where the maturation occurs. The absorption of pre-vitelline material probably occurs in the coelomic fluid; firstly, directly from the fluid, when primary oocytes are clustered with no follicular cell support; posteriorly, secondary oocytes receive follicle cells support and the absorption probably occurs through these cells. In this way, the oogenesis in *L. culveri* from Brazil can be classified as extraovarian type II.

Klesch (1970) studied the same species collected in Texas, USA, and observed that the oogenesis begins in the peritoneal germinative tissue, and oogonia clusters, agglutinated by a basophilic matrix, are released from this tissue. Afterwards, already as primary oocytes, the germ cells release from each other and, individually floating in the coelom, go through the whole vitellogenesis process. All germ cells observed and described by Klesch (1970) are spherical, and the mature oocyte exhibit a thin shell in its external surface. Furthermore, the author did not observe true follicular cells associated and/or attached to germ cells, suggesting that peritoneum cells should play this role. To illustrate the comparison between Klesch´s findings and ours, we provided herein a schematic illustration side-by-side to clearly shown the divergent histological features that take us to our inferences (Figure 6).

In the same way, Florêncio (1999) also observed oocytes floating in the coelom at the beginning of vitellogenesis and in the final phase of this process in individuals identified as *Laeonereis acuta* (Treadwell 1923), collected on the beach of Enseada dos Corais, Pernambuco, Northeast Brazil. This congeneric species is presently considered to be a junior synonym of *L. culveri* (Oliveira 2009; Read & Fauchald 2018), so it can be assumed that it is a different population of *L. culveri* of the Brazilian coast. In the same study, gametes in the proliferative phase (oogonia) were observed in a single individual as cell-agglomerates without the presence of a cellular sheath and the author never mentioned follicular cells associated.

Thus, *L. culveri* specimens from Texas and *L. culveri* specimens from Pernambuco (*L. acuta*) present an extraovarian oogenesis type I, with no follicular cell associated and the yolk precursors probably absorbed directly from coelom. Klesch (1970) suggest also a participation of parenchymal cells in this role. On the other hand, *L. culveri* specimens from São Sebastião (this study) present an extraovarian oogenesis type II with an association of follicular cells, which houses oocytes in clusters until later phases of vitellogenesis. Furthermore, the oocyte morphology and the shell deposition in mature oocytes seem to be very important characters which can operate as reproductive barriers between distinct correlated species. These marked differences among individuals from geographically distinct populations of *L. culveri* suggest the existence of at least two lineages, probably more, on the Atlantic coast of the Americas, reinforcing the indication that it is a case of cryptic species.

Herein, we are considering *L. culveri* from Texas as a different population primarily to fit in our purpose, but also because Klesch (1970) identified these specimens as *L. culveri*. As our goal is to demonstrate the histology of FRS as a good method to complement cryptic species complexes elucidation, it seems to be scientifically valid comparing our results to those found by Klesch (1970), as well as to make use of *L. culveri* barcodes from other regions from USA. In this sense, other authors, such as Oliveira (2009) and Oliveira et al. (2010), performed morphological analysis attempting to diagnose characters variation within several different populations of *Laeonereis* and concluded that *L. culveri* is a truly cosmopolitan species, which shows morphological variability not related to geographical occurrence, rather to environment contamination and fixation techniques. Those findings corroborate Pettibone (1971), in which four species were synonymized with *L. culveri*, also concluding that *L. culveri* is a truly cosmopolitan species, but limited to the North and South Atlantic coast of the Americas (Jesús-Flores et al. 2016).

Our findings regarding the FRS histology and DNA barcode strongly support the hypothesis that *L. culveri* represents a species complex and the histological features of the reproductive structures differ among the specimens of *L. culveri* among the populations herein compared (Klesch 1970, Florêncio 1999, this study). Such differences may also indicate the existence of reproductive barriers between individuals of different populations, therefore possibly revealing a process of speciation.

The COI sequence data obtained for *L. culveri* corroborate the inferences based on the histological observations. The specimens from São Sebastião, Brazil show an intraspecific distance of 16.8% from specimens collected in the Rhode River, USA, also identified as *L. culveri*. This genetic distance is considerably higher than the most frequently observed values for maximum intraspecific distances (about 2 to 3% only) in comprehensive analyses of various invertebrate taxa (Ratnasingham & Hebert 2013), including polychaete fauna (Lobo et al. 2016) which leads to the strong indication that these are two distinct species, and their reproductive features are relevant. Analyses of additional specimens from other localities, particularly from Texas and along the Brazilian coast, are required to gain a comprehensive picture on the taxonomic status of this species, mainly searching for morphological characters that allow species differentiation, as well as for molecular data for species delimitation.

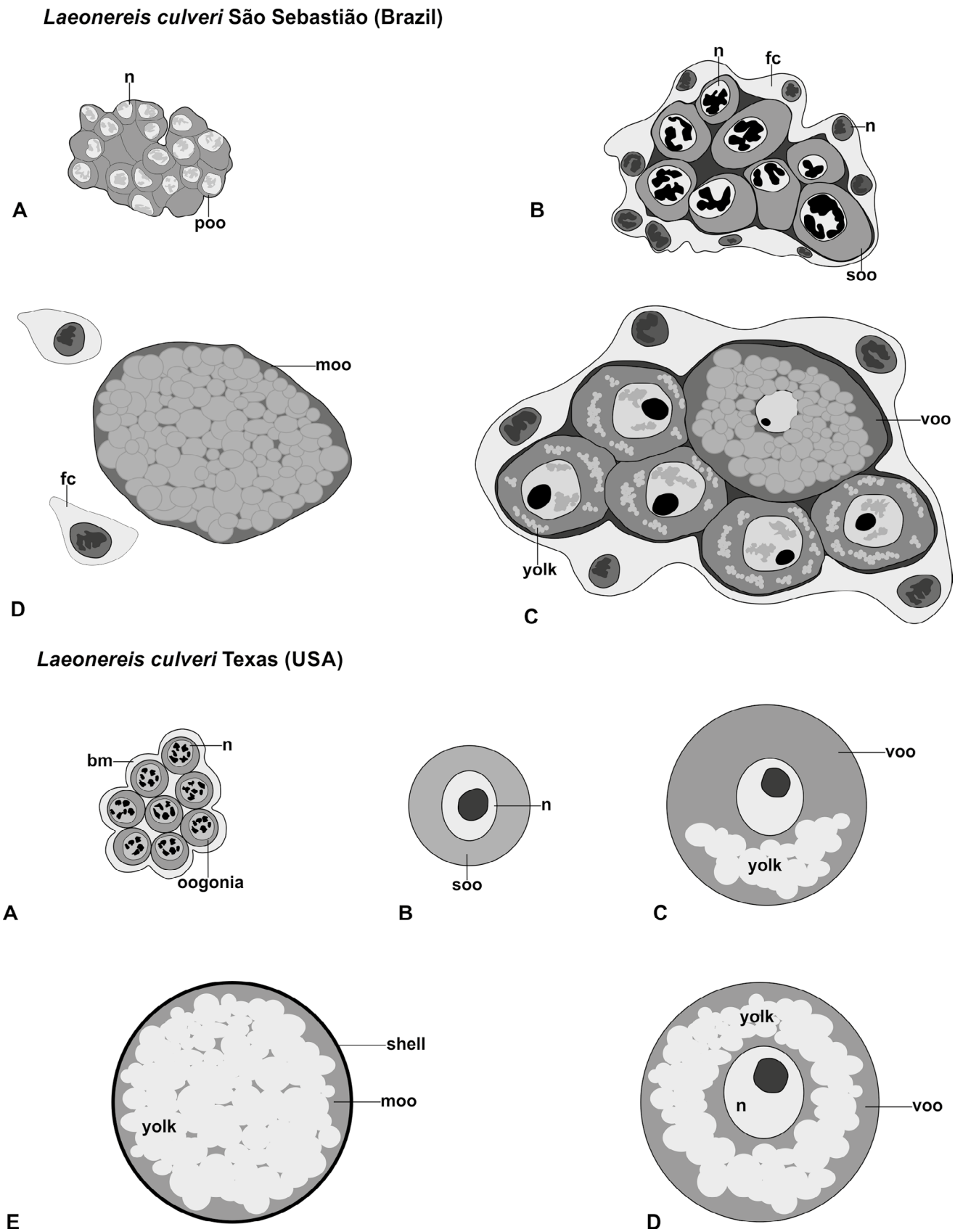

Figure 6. Schematic illustration comparing the oogenesis process in two distinct populations of *Laenereis culveri*. ***Laeonereis culveri*** **from São Sebastião (Brazil): A –** Primary oocytes cluster; **B –** Secondary oocytes clusters wrapped by a follicular cell sheath; **C –** Vitellogenic oocytes inside the follicular cell sheath; **D –** Mature oocyte released from the sheath. ***Laeonereis culveri*** **from Texas (USA): A –** Cluster of oogonia; **B –** Secondary oocyte; **C** and **D –** Vitellogenic oocytes; **E –** Mature oocyte. **bm** = basophilic matrix; **fc** = follicular cell; **moo** = mature oocyte; **n** = nucleus; **poo** = primary oocyte; **soo** = secondary oocytes; **voo** = vitellogenic oocyte.

2. *The genus* Scolelepis: *a well-defined taxa*

The follicular cells of *S. goodbodyi* exhibit a peculiar organization; they are distributed externally along blind-capillaries until reaching the oocytes. These oocytes produce numerous macrovilli (membrane specializations), which allow them to connect with the follicular cells and other tissues, creating a network between blood vessels, follicular cells and oocytes. This complex configuration shows a high-level specialization of the cellular and subcellular structures related to vitellogenesis process, not yet described in the literature for polychaete species.

Richards (1970), with the study of the reproductive biology of *Scolelepis squamata* (Müller, 1806) collected in Barbados (Caribbean), reported similar morphological, histological and physiological aspects to those observed here for *S. goodbodyi*, such as lateral ovary (associated to blood vessels) formed by peritoneal epithelium, as well as oval-shaped oocytes with macrovilli external to the shell zone. However, the author pointed out the occurrence of loose oocytes in the coelomic cavity of the specimens in the final phase of vitellogenesis (*i.e.*, intraovarian type I) whereas an intraovarian type II oogenesis was described here for *S. goodbodyi*. For *S. squamata* there is no report of a network between the oocyte macrovilli and the follicular cells, or even with the intestine and blood vessels (Richards 1970), being a striking feature observed here for *S. goodbodyi*. In this sense, it is supposed that in *S. squamata* the interconnected network that optimizes the vitellogenesis does not occur in any way and the macrovilli function for this species is only to increase the absorption area. This characteristic should be considered as a relevant interspecific variation for species (re)description and differentiation within the genus *Scolelepis*.

Blake & Arnofsky (1999) considered the anatomical position of the ovaries and the ultrastructure of the ovary envelope as the most relevant characters of the female reproductive system for phylogenetic analysis and species differentiation in Spionidae. According to the comparison between *S. squamata* and *S. goodbodyi* regarding the differences of the oogenesis types (intraovarian type I and type II, respectively) and the macrovilli network, these characters should be used for further phylogenetic and/or taxonomic analysis (Richards 1970, Blake & Arnofsky 1999, Eckelbarger 2001). The genetic data obtained show *S. goodbodyi* as a well-defined distinct group, which has a divergence of 19.66% from *S. squamata* from the USA, reinforcing that histological features could be relevant for integrative studies regarding Spionidae.

3. *The genus* Capitella: *messing up minds since the 17th century*

According to Blake (2009), studies carried out by several authors in the last 30-40 years about classification, occurrence and distribution, ecology and morphology of the supposedly cosmopolitan species *Capitella capitata* (Fabricius 1780) have revealed, in fact, a complex of species with a very similar morphology (internal and external). Grassle & Grassle (1976) and Eckelbarger & Grassle (1982, 1983) demonstrated the existence of approximately eight to twelve distinct species occurring on the North American coast. In these studies, the authors recognized and described six sibling species (named *Capitella* sp. I, Ia, II, IIa, III and IIIa) through life history traits and reproductive features, including ovary morphology and oogenesis.

Blake et al. (2009) described and named one of these sibling species, the *Capitella* sp. I, as *Capitella teleta* Blake, Grassle & Eckelbarger 2009, using morphological characters, life history features, and the COI gene sequence. Posteriorly, Tomioka et al. (2016), working with morphological identification and DNA barcode (COI) of the same species, confirmed that *C. teleta* has a real cosmopolitan distribution, as previously supposed, certain phenotypic plasticity and intraspecific variation of a couple of morphological characters. Another sibling species already identified is *Capitella* sp. III, as *Capitella jonesi* (Hartman, 1959) by Eckelbarger & Grassle (1982); however, the other sibling species remain unidentified.

Capitella teleta and *C. jonesi* exhibit a very similar morphology and histology of the reproductive system, except in the number and average size of the oocytes and their yolk composition. However, those characters may vary according to environmental conditions and/or ecological features, such as food-type availability, contaminants, sex ratio and local abundance, demanding an integrative approach, including DNA barcoding, to separate them into distinct species (Eckelbarger 1994, 2001, 2005, Blake & Arnofsky 1999).

Capitella biota was recently described for the Brazilian coast after an extensive review of specimens previously identified as *C. capitata*, which is known to be restricted to its type locality (Greenland, Arctic Circle) (Blake 2009, Tomioka et al. 2016, Silva et al. 2017). The female reproductive system of this species exhibits characteristics similar to *C. teleta* and *C. jonesi* corroborating the hypothesis that it is a highly complex morpho-physiological model within polychaetes (Eckelbarger & Grassle 1982, 1983, Blake et al. 2009, Silva et al. 2017).

The paired ovaries in the reproductive chetigers with sac-like follicles, delimited by a wall of flattened epithelial cells, anchored laterally and dorsally on the body walls and on the septum, respectively, are common characters for the genus *Capitella* and have also been observed in *C. biota, C. teleta* and *C. jonesi*. Nevertheless, it was possible to highlight an important difference between *C. biota* and the other species studied: the ovarian wall isolates the oocytes, housed inside the follicles, since pre-vitellinic stages and being separated from the follicular cells. Eckelbarger & Grassle (1982, 1983) described an antagonistic situation for *C. teleta* (*Capitella* sp. I) and *C. jonesi* (*Capitela* sp. III). In both, the oocytes are surrounded by follicular cells that fill the spaces between the oocytes at different stages of vitellogenesis, and this whole set of germinal and follicular cells are, in turn, packaged in a sheath of epithelial cells (ovarian wall). It is then believed that the shape, distribution and tissue organization of follicular cells, as well as the organization of follicles could represent an interspecific variation that allows the separation of species of the genus *Capitella*.

The COI sequence data presented here for *Capitella* bring relevant findings for the taxonomic issues of this genus:

a) The tree exhibits *C. biota* as a very distinct group from the other species with a genetic distance between 18 and 20% from *C. teleta* and *C. jonesi*;

b) The *Capitella* sp. II (2) and sp. III (3) are the same species, probably *C. jonesi*, justifying why they have almost identical reproductive system and external morphology;

c) *Capitella teleta* from the USA has a genetic distance of almost 18% from *Capitella* sp. II and III (*C. jonesi*).

The differences observed between the ovaries of *C. biota* and the other two congeneric species corroborates that this is indeed a distinct one within the complex, as demonstrated by Silva et al. (2017). In addition, the genetic data shows how intricate is the group, housing at the same time cryptic and cosmopolitan species. Thus, our comparative analysis demonstrates the value of histological features from the FRS to help solving misleading classification and species descriptions within the *Capitella* complex.

4. *The outcome so far*

Given the great variety of characteristics of polychaete female reproductive system, a phylogenetic study based on these characters seems to be unpractical and time-consuming, mainly in a scenario where molecular tools are taking up space in a fast way. Nevertheless, it is likely that such information, when generated through extensive sampling and application of varied techniques, may bring a multi-tool approach to separate correlated species, such as true cryptic and cosmopolitan ones.

The microhabitat of small benthic species, such as the interstitial (meiofauna) and the infauna, has a great influence on its phenotypic modulation, for example, related to the reproductive behavior (cohort, parental care) and such as the morphology of the reproductive system. In this sense, there are indications that this wide variety of reproductive modes and gametogenesis/vitellogenesis types in polychaetes, in some cases, are associated with the environment in which these individuals occur, as well as the overlap of ecological niches (Eckelbarger 2001, Rouse & Pleijel 2001, Katz & Rouse 2013).

From time to time, researchers trying to understand genetic patterns that indicate the occurrence of highly divergent lineages within same species and associated geographic distributions, are appealed to the classical morphological techniques, as well as histological ones, to obtain satisfactory answers (Blake & Arnosfsky 1999, Sato & Nakashima 2003, Lobo et al. 2016;). Lack of diagnostic morphological characters constitutes a major hindrance for the description and widespread acceptance and recognition of the numerous cryptic species of polychaetes and other invertebrates that have been detected over the last years. The "*Laeonereis* complex" is a good example of this. Our study indicates that the histology of ovary and oocytes could constitute a tool that can be added to molecular methodologies, thereby greatly assisting the description, re-description, and systematic analysis of cryptic and pseudo-cryptic species of these annelids, and eventually other invertebrate species.

Acknowledgments

The authors would like to thank IB/UNICAMP, IO/USP and CEBIMar/USP for providing logistic support. In addition, the authors would like to thank the CBMA and the IB-S for the technical support. This work was supported by the FAPESP (Grants nº 2011/50317-5, 2015/25623-6, 2017/06167-5) and CNPq through a productivity grant to A.C.Z.A (306534/2015-0). M.A.L.T was supported by a PhD fellowship (SFRH/BD/131527/2017) from FCT. P.E.V. was supported by a Post-Doctoral Fellowships (BPD1/next-sea/2018, NORTE-01-0145-FEDER-000032). F.O.C. and the University of Minho contribution was supported by the strategic programme UID/BIA/04050/2013 POCI-01-0145-FEDER-007569.

Author Contributions

Bruno R. Sampieri: Substantial contribution on the work conception and design, as well as with the data collection and analysis.

Tatiana M. Steiner: Contributed on data acquisition and text writing.

Camila Fernanda da Silva: Contributed on data acquisition and text writing.

Priscila C. Baroni: Contributed on data acquisition and text writing.

Marcos A. L. Teixeira: Contributed with data analysis and interpretation as well as with text writing.

Pedro E. Vieira: Contributed with data analysis and interpretation as well as with text writing.

Antonia C. Z. Amaral: Contributed substantially to its development.

Filipe O. Costa: Contributed substantially to its development.

Conflicts of Interest

The authors have no conflict of interest to declare.

Data availability

All DNA sequences used in the present study and obtained by the authors through sampling were deposited and published in Genbank and in the Barcode of Life Database (BOLD).

References

Adiyodi, K.G. & Adiyodi, R.G. 1983. Reproductive Biology of Invertebrates. Volume I - Oogenesis, Oviposition and Oosorption. John Wiley and Sons, New York.

Aguado, M.T., Capa, M., Oceguera-Figueroa, A. & Rouse, G.W. 2014. Annelids: Segmented Worms. In The Tree of Life: Evolution and Classification of Living Organisms (P. Vargas & R. Zardoya, eds.). Sinauer Associates, Sunderland, p. 254-269.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410. *doi*:10.1016/S0022-2836(05)80360-2.

Blake, J.A. 2009. Redescription of *Capitella capitata* (Fabricius) from West Greenland and designation of a neotype (Polychaeta, Capitellidae). Zoosymposia. 2:55−80. *doi*:10.11646/zoosymposia.2.1.7.

Blake, J.A. & Arnofsky, P.L. 1999. Reproduction and larval development of the spioniform polychaete with application to systematics and phylogeny. Hydrobiologia. 402:57–106. *doi*:10.1023/A:1003784324125.

Blake, J.A., Grassle, J. P. & Eckelbarger, K.J. 2009. *Capitella teleta*, a new species designation for *Capitella* sp . I, with a review of the literature for confirmed records. *Zoosymposia*. 2:25–53. *doi*:10.11646/zoosymposia.2.1.6.

Carr, C.M., Hardy, S.M., Brown, T.M., Macdonald, T.A., & Hebert, P.D.N. 2011. A Tri-Oceanic Perspective: DNA Barcoding reveals geographic structure and cryptic diversity in Canadian polychaetes. PLoS. One. 6:1-12. *doi*:10.1371/journal.pone.0022232

Costa, F.O. & Antunes, P.M. 2012. The contribution of the Barcode of Life Initiative to the discovery and monitoring of biodiversity. In Natural resources, sustainability and humanity: A comprehensive view (A. Mendonca, A. Cunha & R. Chakrabarti eds.). Springer, Dordrecht. *doi*:10.1007/978-94-007-1321-5_4

Eckelbarger, K.J. 1994. Diversity of metazoan ovaries and vitellogenic mechanisms: implication for life history theory. P. Biol. Soc. Wash. 107:193–218.

Eckelbarger, K.J. 2001. Oogenesis. In Reproductive Biology and Phylogeny of Annelida (G. W. Rouse & F. Pleijel eds.). Oxford University Press, London.

Eckelbarger, K.J. 2005. Oogenesis and oocytes. Hydrobiologia. 535:179–198. *doi*:10.1007/s10750-004-4397-y.

Eckelbarger, K.J. & Grassle, J.P. 1982. Ultrastructure of the ovary and oogenesis in the polychaete *Capitella jonesi* (Hartman, 1959). J. Morphol. 171:305–320. *doi*:10.1002/jmor.1051710306

Eckelbarger, K.J. & Grassle, J.P. 1983. Ultrastructure differences in the eggs and ovarian follicle cells of *Capitella* (Polychaeta) sibling species. Bio. Bull. 165:379–393. *doi*:10.2307/1541203

Fabricius O. 1780. Fauna Groenlandica, systematica sistens, Animalia Groenlandiae occidentalis hactenus indagata, quad nomen specificum, triviale, vernaculumque; synonyma auctorum plurium, descriotionem, locum, victum, generationem, mores, usum, capturamque singuli; prout detegendi occasio fuit,maximaque parti secundum proÂprias observationes. Copenhagen & Leipzig.

Faroni-Perez, L. & Zara, F. J. 2014. Oogenesis in *Phragmatopoma* (Polychaeta: Sabellariidae): Evidence for morphological distinction among geographically remote populations. Mem. Mus. Vic. 71:1447–2554.

Florêncio, M.S. 1999. Ciclo reprodutivo e histologia do trato digestivo de Laeonereis acuta (Treadwell, 1923) da praia de Enseada dos Corais - Pernambuco. Dissertação de Mestrado, Universidade Federal do Pernambuco, Recife.

Garraffoni, A.R.S., Yokoyama, L.Q. & Amaral, A.C.Z. 2014. The gametogenic cycle and life history of *Nicolea uspiana* (Polychaeta: Terebellidae) on the south-east coast of Brazil. J. Mar. Biol. Assoc. UK. 94:925–933. *doi*:10.1017/S0025315414000149

Glover, A.G., Goetze, E., Dahlgren, T.G., Smith, C.R. 2005. Morphology, reproductive biology and genetic structure of the whale-fall and hydrothermal vent specialist, Bathykurila guaymasensis Pettibone, 1989 (Annelida: Polynoidae). Mar. Ecol. 26:223-234. doi:10.1111/j.1439-0485.2005.00060.x

Grassle, J.P. & Grassle, J.F. 1976. Sibling species in the marine pollution indicator *Capitella* (Polychaeta). Science. 192:567–569. *doi*:10.1126/science.1257794

Hartman, O. 1959. Capitellidae and Nereidae (Marine annelids) from gulf of Florida, with a review of freshwater Nereidae. B. Mar. Sci. 9:153-168.

Hajibabaei, M., Smith, M.A., Janzen, D.H., Rodriguez, J.J., Whitfield, J.B. & Hebert, P.D.N. 2006. A minimalist barcode can identify a specimen whose DNA is degraded. Mol. Ecol. Notes. 6:959-964. *doi*:10.1111/j.1471-8286.2006.01470.x

Hebert, P. D.N, Cywinska, A., Ball, S. L. & deWaard, J.R. (2003). Biological identifications through DNA barcodes. P. Roy. Soc. B-Biol. Sci. 270:313–321. *doi*:10.1098/rspb.2002.2218

Jesús-Flores, C., Salazar-Gonzáles, S.A. & Salazar-Vallejo, A.S. 2016. Morphological distinction between estuarine polychaetes: *Laeonereis culveri* and *L. nota* (Phyllodocida: Nereididae). Rev. Biol. Trop., 64:189-201.

Jones, M.L. 1962. On some polychaetous annelids from Jamaica, the West Indies. *B. Am. Mus. Nat. Hist.* 124: 169-212.

Katz, S. & Rouse, G. W. 2013. The reproductive system of *Osedax* (Annelida, Siboglinidae): ovary structure, sperm ultrastructure, and fertilization mode. Invertebr. Biol. 132:368–385. *doi*:10.1111/ivb.12037

Kinberg, J.G.H. 1866 (or 1867). Annulata nova. [Continuatio.]. *Öfversigt af Königlich Vetenskapsakademiens förhandlingar, Stockholm,* 23:337-357.

Klesch, W.L. 1970. The reproductive biology and larval development of *Laeonereis culveri* Webster (Polychaeta; Nereididae). Contrib. Mar. Sci. 15:71-75.

Knebelsberger, T., Dunz, A.R., Neumann, D., Geiger, M.F. 2015. Molecular diversity of Germany´s freshwater fishes and lampreys assessed by DNA barcoding. Mol. Ecol. Resour. 15:562-572. *doi*: 10.1111/1755-0998.12322

Kumar, S., Stecher, G., & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33:1870–1874. *doi*:10.1093/molbev/msw054

Langeneck, J., Del Pasqua, M., Licciano, M., Giangrande, A., Musco, L. 2020. Atypical reproduction in a syllid worm: the stolon of *Syllis rosea* (Annelida, Syllidae) takes care of its offspring. J. Mar. Biol. Assoc. UK., 1-7. https://doi.org/10.1017/S0025315420000119

Lobo, J., Teixeira, M.A.L., Borges, L.M.S., Ferreira, M.S.G., Hollatz, C., Gomes, P.A., Sousa, R., Ravara, A., Costa, M.H., & Costa, F.O. 2016. Starting a DNA barcode reference library for shallow water polychaetes from the southern European Atlantic coast. Mol. Ecol. 16:288-313. *doi*:10.1111/1755-0998.12441

MacCord, F.S., Amaral, A.C.Z. 2007. The reproductive cycle of *Scolelepis goodbodyi* (Polychaeta, Spionidae). Mar. Biol. 151:1009–1020. *doi*:10.1007/s00227-006-0540-9

Martin, D., Gil, J., Zanol, J., Meca, M.A., Portela, R.P. 2020. Digging the diversity of iberian bait worms *Marphysa* (Annelida, Eunicidae). PLoS One, 15(1):e0226749. https://doi.org/10.1371/journal.pone.0226749

Meißner, K. & Götting, M. 2015. Spionidae (Annelida: "Polychaeta": Canalipalpata) from Lizard Island, Great Barrier Reef, Australia: the genera Malacoceros, Scolelepis, Spio, Microspio, and Spiophanes. Zootaxa, 4019: 378-413. http://dx.doi.org/10.11646/zootaxa.4019.1.15

Müller, O.F. 1806. Zoologia Danica seu animalium Daniae et Norvegiae rariorum ac minus notorum descriptiones et historia. *Havniae [Copenhague], N. Christensen:* Volumen quartum: [1-5], 1-46, p. 121-160

Nunes, F.L.D., Wormhoudt, A.V., Faroni-Perez, L., Fournier, J. 2017. Phylogeography of the reef-building polychaetes of the genus *Phragmatopoma* in the western Atlantic Region. J. Biogeogr., 44:1612-1625. doi: 10.1111/jbi.12938.

Nygren, A. 2014. Cryptic polychaete diversity: a review. Zool. Scr., 43, 172-183. *doi*:10.1111/zsc.12044

Oliveira, V. M. 2009. Variabilidade morfológica de *Laeonereis* (Hartman, 1945) (Polychaeta: Nereididae) ao longo do Atlântico Ocidental. Doctorate Thesis, Universidade Federal do Paraná, Curitiba.

Oliveira, V.M., Santos, C.S.G., Lana, P.C. & Camargo, M.G. 2010. Morphological variations caused by fixation techcniques may lead to taxonomic confusion in *Laeonereis* (Polychaeta: Nereididae). Zoologia-Curitba, 27:146-150.

Omena, E.P. & Amaral, A.C.Z. 2001. Morphometric study of the nereidid *Laeonereis acuta* (Annelida: Polychaeta). J. Mar. Biol. Assoc. UK. 81:423-426. *doi*:10.1017/S0025315401004040

Pettibone, M.H. 1989. Polynoidae and Sigalionidae (Polychaeta) from the Guaymas Basin, with descriptions of two new species, and additional records from hydrothermal vents of the Galapagos Rift, 21ºN, and seep-sites in the Gulf of Mexico (Florida and Louisiana). *P. Biol. Soc. Wash.* 102(1): 154-168.

Pettibone, M.H. 1971. Revision of some species referred to *Leptonereis*, *Nicon*, and *Laeonereis* (Polychaeta: Nereididae). Smithson. Contr. Zool. 104:1–53.

Radulovici, A.E., Sainte-Marie, B. & Dufresne, F. 2009. DNA barcoding of marine crustaceans from the Estuary and Gulf of St Lawrence: a regional-scale approach. Mol. Ecol. Resour. 9:181-187. *doi*:10.1111/J.1755-0998.2009.02643.x

Ratnasingham, S. & Hebert, P.D.N. 2007. BOLD: The Barcode of Life Data System. Mol. Ecol. Notes. 7:355–364. *doi*:10.1111/j.1471-8286.2006.01678.x

Ratnasingham, S. & Hebert, P.D.N. 2013. A DNA-based registry for all animal species: the barcode index number (BIN) system. PLoS. One. 8:e66213. *doi*:10.1371/journal.pone.0066213

Raupach, M.J., Barco, A., Steinke, D., Beermann, J., Laakmann, S., Mohrbeck, I., Neumann, H., Kihara, T.C., Pointner, K., Radulovici, A., Segelken-Voigt, A., Wesse, C. & Knebelsberger, T. 2015. The Application of DNA Barcodes for the Identification of Marine Crustaceans from the North Sea and Adjacent Regions. PLoS One. 10:e0139421. doi:10.1371/journal.pone.0139421.

Read, G. & Fauchald, K. 2018. World Polychaeta database. *Laeonereis culveri* (Webster, 1879). http://www.marinespecies.org/aphia.php?p=taxdetails&id=333727. (Accessed 12 December 2019).

Richards, S.L. 1970. Spawning and reproductive morphology of *Scolelepis squamata* (Spionidae: Polychaeta). Can. J. Zool. 48:1369–1379. *doi*:10.1139/z70-234

Rouse, G. W. & Pleijel, F. 2001. Polychaete*s*. Oxford University Press, New York.

Sato, M. & Nakashima, A. 2003. A review of Asian *Hediste* species complex (Nereididae, Polychaeta) with descriptions of two new species and a redescription of *Hediste japonica* (Izuka, 1908). Zool. J. Linn. Soc-Lond. 137:403-445. *doi*:10.1046/j.1096-3642.2003.00059.x

Schroeder, P.C. & Hermans, C. O. 1975. Annelida: Polychaeta. In Reproduction of Marine Invertebrates. Volume III, Annelids and Echiurans (A.C. Giese & J.S. Pearse eds.). Academic Press, New York.

Silva, C.F., Seixas, V.C., Barroso, R., Di Domenico, M., Amaral, A.C.Z. & Paiva, P.C. 2017. Demystifying the *Capitella capitata* complex (Annelida , Capitellidae) diversity by morphological and molecular data along the Brazilian coast. PLoS One. 12. *doi*:10.1371/journal.pone.0177760

Teixeira, M.A., Vieira, P.E., Pleijel, F., Sampieri, B.R., Ravara, A., Costa, F.O., Nygren, A. 2020. Molecular and morphometric analyses identify new lineages within a large *Eumida* (Annelida) species complex. Zool. Scr. 49:222-235. https://doi.org/10.1 111/zsc.12397

Thompson, J.D., Higgins, D.G. & Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680. *doi*:10.1093/nar/22.22.4673

Tomioka, S., Kondoh, T., Sato-Okoshi, W., Ito, I., Kakui, K. & Kajihara, H. 2016. Cosmopolitan or Cryptic Species? A Case Study of *Capitella teleta* (Annelida: Capitellidae). Zool. Sci. 33:545-554. *doi*:10.2108/zs160059

Treadwell, A.L. 1923. Duas novas espécies de Annelidos Polychetos do Genero Nereis. Revista do Museu Paulista, 13:1-10.

Webster, H.E. 1879. The Annelida Chaetopoda of New Jersey. *Annual Report of the New York State Museum of Natural History.* 32:101-128.

Wilson, W.H. 1991. Sexual reproductive modes in polychaetes: classification and diversity. B. Mar. Sci. 48:500–516.

Received: 15/01/2020
Revised: 10/06/2020
Accepted: 17/06/2020
Published online: 31/07/2020