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Interspecific variability in internal anatomy in *Terebellides* Sars, 1835 (Annelida, Trichobranchidae) revealed with micro-CT

María Barroso [a,*], María Candás [b], Juan Moreira [c], Julio Parapar [a]

[a] Departamento de Bioloxía, Universidade da Coruña, Spain
[b] Rede de Estacións Biolóxicas da Universidade de Santiago de Compostela (REBUSC), Estación de Bioloxía Mariña da Graña, Universidade de Santiago de Compostela, Rúa da Ribeira 1-4, A Graña-Ferrol, Spain
[c] Departamento de Biología (Zoología) & Centro de Investigación en Biodiversidad y Cambio Global (CIBC-UAM), Facultad de Ciencias, Universidad Autónoma de Madrid, Spain

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ABSTRACT

The number of new species described of the genus *Terebellides* Sars, 1835 (Annelida, Trichobranchidae) in the eastern Atlantic Ocean has greatly increased in the last years. Eleven of these species, all originally described in NE Atlantic Ocean, from the Barents Sea to the Gulf of Guinea, have been studied using micro-computed tomography (micro-CT) thus revealing interspecific differences in several elements of the internal anatomy such as the proportion between stomach regions and size of nephridia, and in the number of lamellae in the digestive gland. Two stomach morphotypes have been distinguished regarding differences in relative length between regions, as well as two morphotypes of nephridia according to their size and wall thickness, and four types of the digestive gland regarding their number of lamellae. Differences in nephridia sizes is the only character apparently related to the geographic distribution of species (i.e. NE Atlantic *vs.* Gulf of Guinea) but examination of more taxa is needed. A third distinct region was distinguished in the stomach, named here as mid stomach, that differs from the fore and hind stomach in epithelia thickness and by lacking connective muscular sheath. The potential of the micro-CT technique in the search for internal anatomical characters with taxonomic value is discussed.

1. Introduction

The genus *Terebellides* Sars, 1835 is the most speciose in the family Trichobranchidae Malmgren, 1866 with up to 85 valid species (Read & Fauchald, 2023). Diagnostic characters correspond mostly to external body features such as branchial shape, ventral pigmentation of anterior thoracic chaetigers and features of thoracic and abdominal uncini (Parapar et al., 2020a, b, Barroso et al., 2022). In the last decade, 29 species have been described based on external morphology alone (Schüller & Hutchings, 2012, 2013, Parapar et al., 2013, 2016a, b, c, 2020b, Hutchings et al., 2015, Hsueh & Li, 2017, Zhang & Hutchings, 2018). However, molecular analysis of a large number of specimens carried out by Nygren et al. (2018) and Lavesque et al. (2019) revealed an unexpected high diversity of species in the Northeast Atlantic Ocean that resulted in the description of 13 new species so far (Lavesque et al., 2019; Parapar et al., 2020a; Barroso et al., 2022).

The internal anatomy of *Terebellides* has been studied by Steen (1883), Wirén (1885), Hessle (1917), Michel et al. (1984), Williams (1984) and Penry & Jumars (1990). They reported a highly regionalised digestive system composed of mouth, oesophagus, fore stomach (surrounded by a digestive gland), hind stomach, fore and hind intestine. A pair of nephridia was also reported in the thoracic region ventrally to oesophagus, as well as a longitudinal dorsal blood vessel. However, the potential interspecific variability of any of these characters was not assessed until Williams (1984) described *Terebellides distincta* Williams, 1984 based on the different shape and length of the fore stomach compared to the hind stomach. Williams (1984) also stated that the two stomach regions were similar to each other in shape and length in specimens identified as *Terebellides stroemii*. Nevertheless, following work did not pay attention to internal anatomy features that could have potential taxonomic value.

X-ray micro-computed tomography (micro-CT) is a non-invasive technique based on computed tomography originally developed to examine human three-dimensional bone structure (Feldkamp et al.,

* Corresponding author.
E-mail address: maria.p.barroso@udc.es (M. Barroso).

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1989). This anatomical technique allows studying and reconstructing the internal anatomy without deterioration of the specimen and creating 3D virtual models from the images obtained (Feldkamp et al., 1989). Currently, micro-CT is also being used in the description of new taxa and to search for new potential taxonomical characters of the internal anatomy in invertebrates, i.e. taxa that lack reliable external morphological characters or taxa where descriptions are mainly based on internal anatomy such as molluscs (Marcondes Machado et al., 2019; Sumner-Rooney et al., 2019; Martínez-Sanjuán et al., 2022), arthropods (Schoborg et al., 2019; Killiny & Brodersen, 2022) and echinoderms (Ziegler, 2019; Samyn et al., 2021). Regarding the genus *Terebellides*, micro-CT examination has been used to describe the gross internal anatomy of *T. stroemii*, *T. shetlandica* and *T. persiae* (Parapar & Hutchings, 2014, Parapar et al., 2016a, c).

The main aim of this paper is to study the internal anatomy of several species of *Terebellides* from eastern Atlantic using the micro-CT technique, in order to look for new internal characters that may also serve to characterise species besides external body characters. Finally, we discuss whether these characters may be related to geographic or bathymetric patterns of distribution.

2. Materials and methods

Twenty-five specimens belonging to eleven species of genus *Terebellides* have been examined, all originally described in NE Atlantic (NEA), from the Barents Sea and North Sea to the Gulf of Guinea (GGA) (Fig. 1). The studied species from the Northeast Atlantic (8 in total) correspond to *T. atlantis* Williams, 1984 (2 specimens: SMF30852, ZMBN116466), *T. europaea* Lavesque, Hutchings, Daffe, Nygren & Londoño-Mesa, 2019 (3 spec.: GNM14625, GNM15117, ZMBN116342), *T. gracilis* Malm, 1874 (2 spec.: GNM15111, ZMBN116276), *T. lavesquei* Barroso, Moreira, Capa, Nygren & Parapar, 2022 (2 spec.: ZMBN116321, ZMBN116326), *T. norvegica* Parapar, Capa, Nygren & Moreira, 2020 (3 spec.: GNM15133, ZMBN116378, ZMBN116380), *T. shetlandica* Parapar, Moreira & O'Reilly, 2016 (3 spec.: NMS. Z2013.07.01, ZMBN116221, ZMBN116227), *T. stroemii* Sars, 1835 (1 spec.: NHMOC5898) and *T. williamsae* Jirkov, 1989 (3 spec.: GNM15108, ZMBN116246, GNM15109). The species examined from the Gulf of Guinea (3 in total) were *T. fauveli* Parapar, Martin & Moreira, 2020 (2 spec.: NHMD1235440, NHMD1235441), *T. kirkegaardi* Parapar, Martin & Moreira, 2020 (2 spec.: NHMD1235438, NHMD1235439) and *T. nkossa* Parapar, Martin & Moreira, 2020 (2 spec.: MNCN16.01/90300, MNCN16.01/90301).

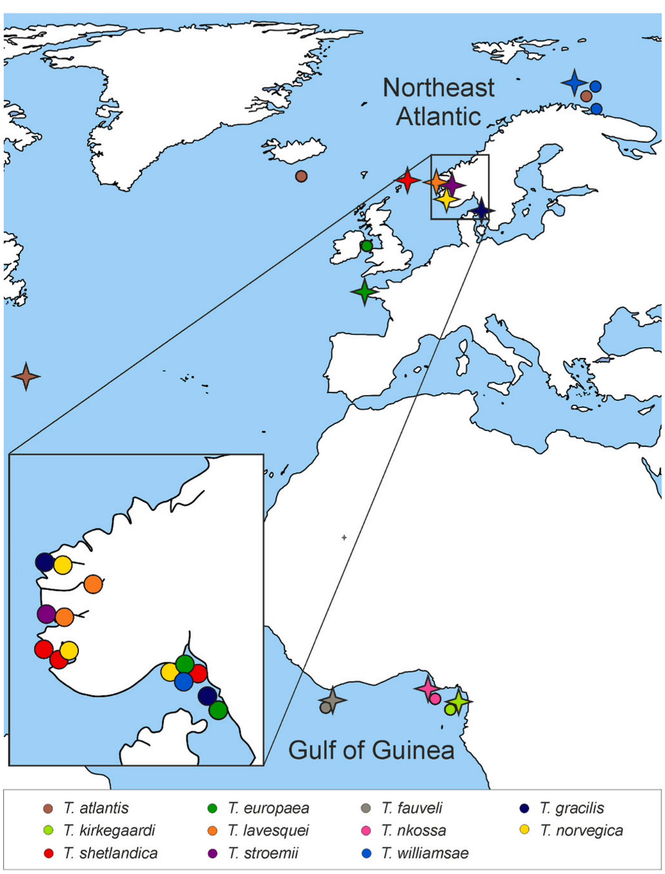

Fig. 1. Sampling localities in the eastern Atlantic Ocean where the studied specimens of *Terebellides* were collected. Stars mark location of the type locality for each species.

Before micro-CT study, some specimens (NHMD1235440, NHMD1235441, NHMD1235438, NHMD1235439, MNCN16.01/90300, MNCN16.01/90301 and NHMOC5898) were initially fixed in formalin, preserved in 70% ethanol while others were fixed and preserved in ethanol 96%. In both cases, specimens were dehydrated in successive baths of 80%, 90% and 96% ethanol, then immersed 2 h in hexamethyldisilazane and allowed to air dry overnight (Parapar et al., 2019). No staining was used. Specimens were then scanned with a microtomograph Skyscan 1172 at the Estación de Bioloxía Mariña da Graña, Universidade de Santiago de Compostela, Spain (REBUSC-EBMG, USC), using the following parameters: 40 kV, 250 μA, unfiltered, image pixel size between 1.49 and 6.89 μm (1.49 μm: ZMBN116466 and NMS. Z2013.07.01; 1.70 μm: SMF30852; 1.97 μm: GNM15109; 2.97 μm: MNCN16.01/90300; 2.98 μm: ZMBN116246; 3.18 μm: ZMBN116276; 3.59 μm: MNCN16.01/90301; 3.79 μm: ZMBN116221; 3.94 μm: ZMBN116380; 4.07 μm: ZMBN116227; 5.03 μm: ZMBN116326; 5.08 μm: ZMBN116321; 5.09 μm: NHMOC5898; 5.50 μm: GNM15117 and GNM15133; 5.57 μm: GNM14625; 6 μm: NHMD1235439; 6.04 μm: ZMBN116342; 6.07 μm: NHMD1235440, NHMD1235441 and GNM15111; 6.55 μm: NHMD1235438; 6.79 μm: ZMBN116378; 6.89 μm: GNM15108) and no camera binning. Images obtained were reconstructed with the NRecon software (Bruker, Belgium) and cleaned with CT Analyzer software (Bruker, Belgium); to visualise the data, DataViewer and CTVox softwares (Bruker, Belgium) were used.

The following measurements were taken in each specimen with DataViewer: 1) nephridial wall thickness, 2) fore stomach: length and wall thickness, 3) digestive gland: length, width and height, 4) mid stomach: length and wall thickness, 5) hind stomach: length and wall thickness, and 6) fore intestine wall thickness. These measurements were taken to search for potential differences between species and whether they could be used to discriminate among species. Body width was always measured at segment 3. Nevertheless, the examined specimens come from different sampling programmes (e.g., benthic monitoring, collection of specimens for molecular analyses) and those were not properly prepared for eventual micro-CT examination. Therefore, the digestive tract of several specimens presents large amounts of sediment (e.g., fore intestine) that prevented to obtain proper images and measurements; these specimens were not treated in any special way.

3. Results

The anatomical elements studied species were: body wall musculature, nephridia, digestive system, circulatory system and nervous system (Figs. 2–7 and Supp. Figs. 1–11). Those best visualised were the nephridia and the regions of the digestive system: mouth, oesophagus, fore and hind stomach, and fore and hind intestine. Furthermore, a new stomach region has been distinguished, named here as mid stomach. It is connected to the digestive gland and is distinguished from neighbouring regions by the different thickness of the epithelium; it also differs from the fore stomach by lacking the connective muscular sheath. This new stomach region is present in all specimens studied but it could only be properly measured in those with an empty digestive tract (Fig. 4A and Supp. Figs. 8A and 11A).

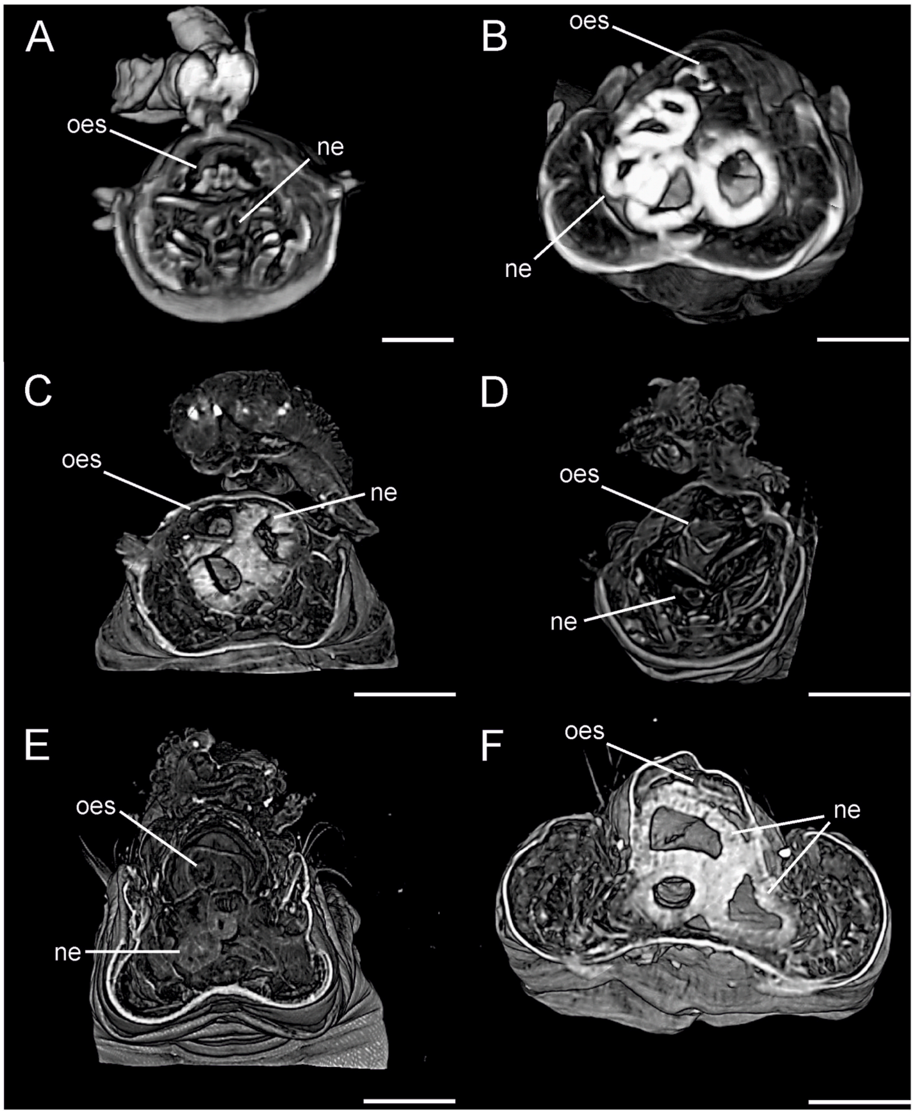

Fig. 2. Micro-CT 3D volume renderings of transversal sections of anterior thoracic region of several *Terebellides* species. (A) *T. fauveli* (NHMD1235440); (B) *T. gracilis* (GNM15111); (C) *T. lavesquei* (ZMBN116321); (D) *T. nkossa* (MNCN16.01/90300); (E) *T. norvegica* (ZMBN116378); (F) *T. shetlandica* (ZMBN116221). Scale bars: A = 250 μm; B–F = 500 μm.
Abbreviations: ne – nephridia; oes – oesophagus.

The arrangement of the body wall musculature, circulatory and nervous systems of the studied species agrees with those previously described for the genus and apparently does not show relevant interspecific differences. All species have a well-developed ventral longitudinal musculature (Fig. 5B and C) much more developed than the dorsal one (Supp. Figs. 4 and 10), a longitudinal dorsal blood vessel close to the digestive tract that is especially evident dorsally to oesophagus and stomach (Fig. 4D and E and Supp. Figs. 1C, 4C, D, 5C, 7B, 9B, 10A, C–E, 11C), and a ventral nerve cord of fused paired ganglia (Fig. 5B and C and Supp. Figs. 11D and E). Gonads were not observed but oocytes were identified in the coelomic cavity of some specimens (Supp. Fig. 6A, C–E).

The nephridia and the digestive tract were present in all the specimens studied and showed evident interspecific differences. Therefore, a description of each species focused on these characters is provided below.

3.1. Anatomy of species

3.1.1. *Terebellides atlantis* Williams, 1984 (Fig. 7A–D; Supp. Figs. 1A–E; Supp. Table 1)

Material examined: 2 specimens. SMF30852: 16 mm long, 0.52 mm wide; ZMBN116466: 13 mm long, 0.34 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity in the thoracic region totally occupied by a pair of nephridia and by the digestive tract in the rest of the body. Nephridia wall thickness ranges from 35 to 56 μm (Supp. Figs. 1A and B). Mouth opens into oesophagus (Supp. Figs. 1A and B) which leads to fore stomach (length: 860 and 575 μm, respectively; wall thickness: 54.3 and 26.8 μm); the former is ventrally surrounded by a voluminous digestive gland (length: 896 and 387.5 μm; width: 643 and 244 μm; height: 333 and 250 μm) (Fig. 7B and C; Supp. Fig. 1C) provided with 3 lamellae (Fig. 7D). Fore stomach continues in a short mid stomach (length: 161 μm -only one measured-; wall thickness: 40.7 and 32.79 μm) to which the digestive gland is connected. Mid stomach followed by hind stomach (length: 1100 and 785 μm; wall thickness: 48.3 and 44.4 μm), the latter being about as long as fore + mid stomach (Supp. Figs. 1A and D). Fore intestine (wall thickness: 37.8 and 33.7 μm) connects with hind intestine (Supp. Fig. 1E).

3.1.2. *Terebellides europaea* Lavesque, Hutchings, Daffe, Nygren & Londoño-Mesa, 2019 (Fig. 7A–D; Supp. Figs. 2A–E; Supp. Table 1)

Material examined: 3 specimens. GNM14625: 21 mm long, 1.27 mm wide; GNM15117: 30 mm long, 3.22 mm wide; ZMBN116342: 26 mm long, 3.33 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity in the thoracic region totally occupied by a pair of nephridia and by the digestive tract in the rest of the body.

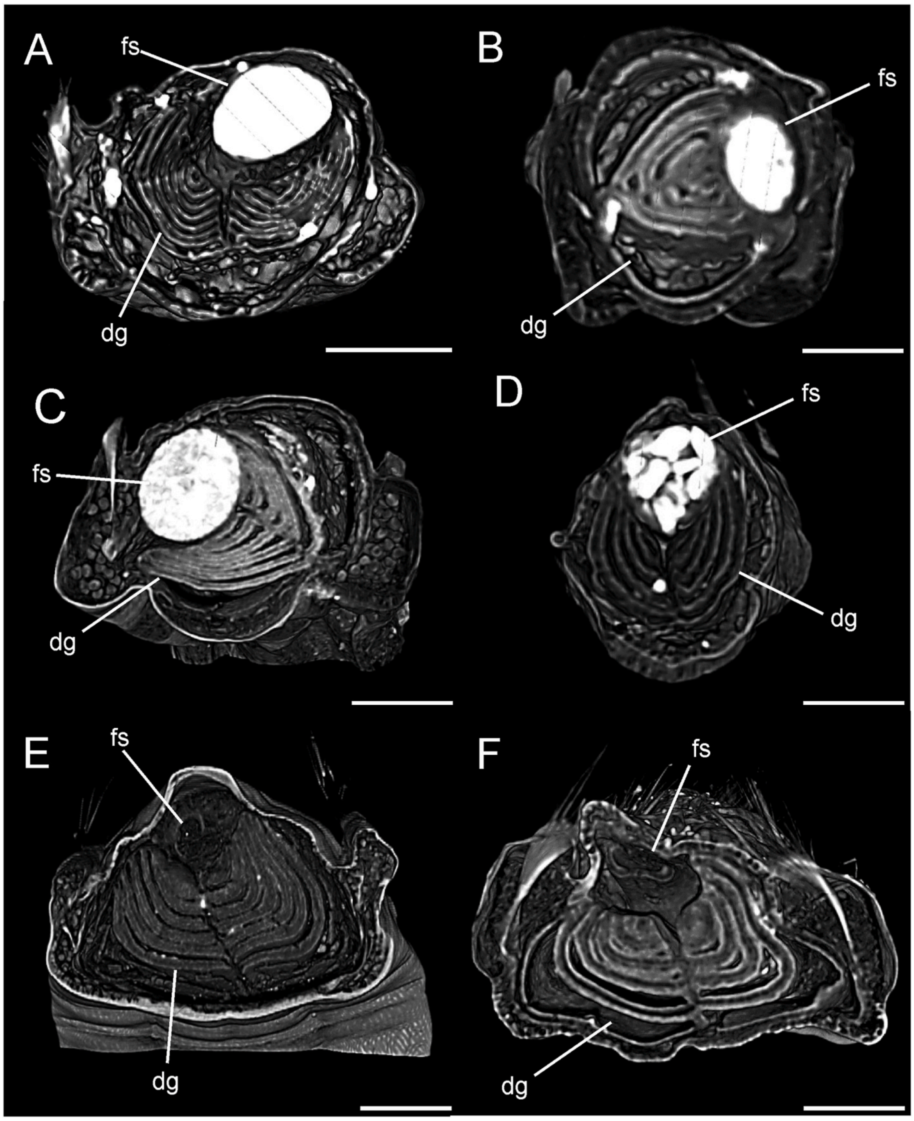

Fig. 3. Micro-CT 3D volume renderings of transversal sections of posterior thoracic region of several *Terebellides* species. (A) *T. fauveli* (NHMD1235440); (B) *T. gracilis* (GNM15111); (C) *T. lavesquei* (ZMBN116321); (D) *T. nkossa* (MNCN16.01/90300); (E) *T. norvegica* (ZMBN116378); (F) *T. shetlandica* (ZMBN116221). Scale bars: A–F = 500 μm. Abbreviations: dg – digestive gland; fs – fore stomach.

Nephridia wall thickness ranges from 78 to 93.5 μm (Supp. Figs. 2A and B). Mouth opens into oesophagus (Supp. Fig. 2B) which leads to fore stomach (length: 1714, 3460 and 1516 μm, respectively; wall thickness: 122.5, 275 and 193.3 μm); the latter is surrounded ventrally by a voluminous digestive gland (length: 2605, 2950.5 and 2139 μm; width: 1414, 1930 and 2417 μm; height: 712, 1760 and 1934 μm) (Fig. 7B and C; Supp. Fig. 2C) that bears 6 lamellae (Fig. 7D). Fore stomach continues in a short mid stomach to which the digestive gland is connected. Mid stomach followed by hind stomach (length: 2059, 3945 and 1898 μm; wall thickness: 160.9, 184.8 and 89.4 μm) is about as long as fore + mid stomach (Supp. Figs. 2A and D). Fore intestine (wall thickness: 135, 181.5 and 94.5 μm) connects with hind intestine (Supp. Fig. 2E).

3.1.3. *Terebellides fauveli Parapar, Martin & Moreira, 2020 (Fig. 2A, 3A, 7A–D; Supp. Figs. 3A–E; Supp. Table 1)*

Material examined: 2 specimens. NHMD1235440: 24 mm long, 0.84 mm wide; NHMD1235441: 30 mm long, 1.01 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity is filled by the digestive tract in the posterior region of the body. Nephridia wall thickness ranges from 24 to 30 μm (Fig. 2A; Supp. Figs. 3A and B). Mouth opens into oesophagus (Supp. Fig. 3B) which leads to fore stomach (length: 1456 and 1335 μm, respectively; wall thickness: 97.1 and 66.7 μm); the latter is surrounded ventrally by a voluminous digestive gland (length: 1468.4 and 1322.8 μm; width: 1092.2 and 770.6 μm; height: 740.3 and 788.8 μm) (Fig. 7B and C; Supp. Fig. 3C) that bears 8 lamellae in one specimen (Fig. 3A and 7D). Fore stomach continues in a short mid stomach to which the digestive gland is connected. Mid stomach followed by hind stomach (length: 1954 and 1140.7 μm; wall thickness: 81 and 66.7 μm) is about as long as fore + mid stomach (Supp. Figs. 3A and D). Fore intestine could not be measured due to the large amount of sediment within (Supp. Fig. 3E).

3.1.4. *Terebellides gracilis Malm, 1874 (Fig. 2B, 3B, 7A–D; Supp. Figs. 4A–E; Supp. Table 1)*

Material examined: 2 specimens. GNM15111: 29 mm long, 1.09 mm wide; ZMBN116276: 15 mm long, 0.61 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity in the thoracic region is totally filled by a pair of nephridia and by the digestive tract in the rest of the body. Nephridia wall thickness ranges from 40 to 140 μm (Fig. 2B; Supp. Figs. 4A and B). Mouth opens into oesophagus (Supp. Fig. 4B) which leads to fore stomach (length: 2087 and 1031.3 μm, respectively; wall thickness: 109.2 and 108.22 μm); the latter is surrounded ventrally by a voluminous digestive gland (length: 1656.5 and 1305 μm; width: 1165 and 509.3 μm; height: 849 and 446.6 μm) (Fig. 7B and C; Supp. Fig. 4C) that

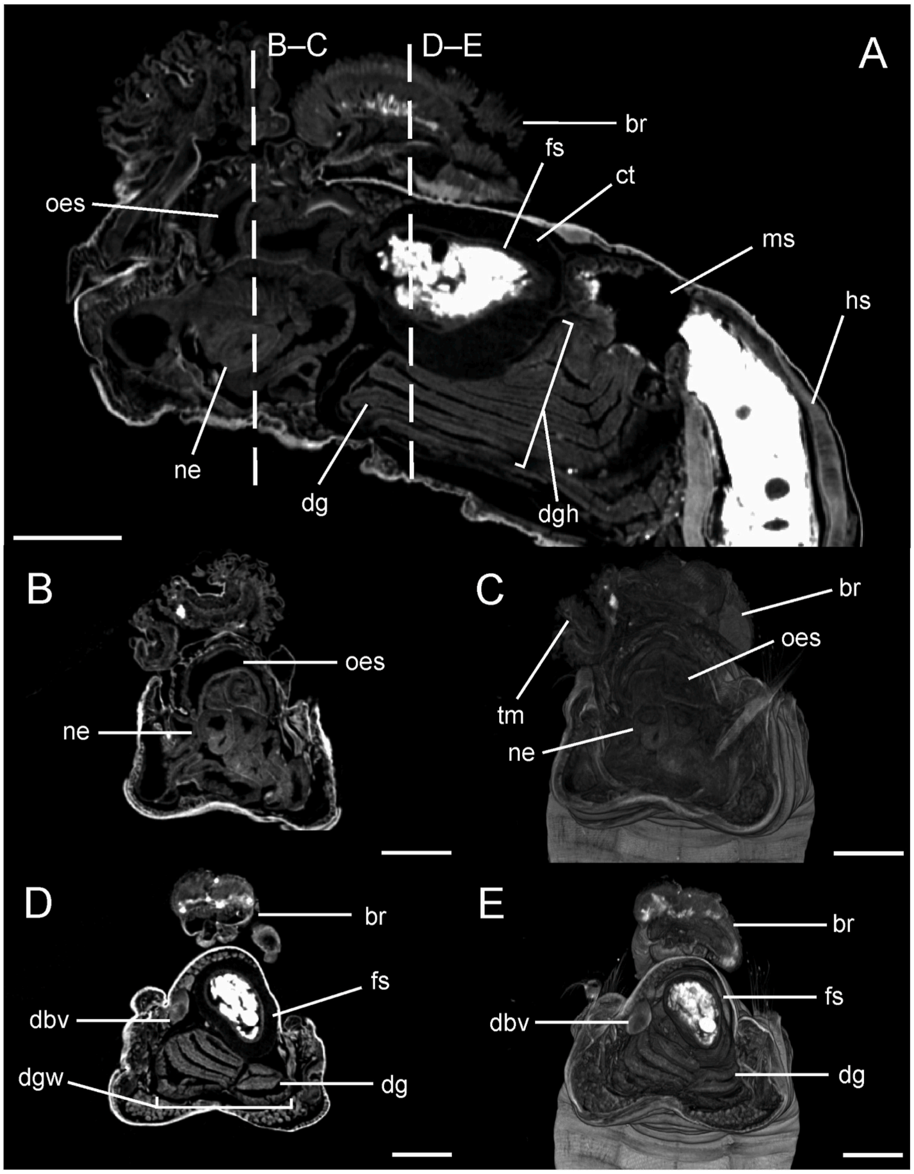

Fig. 4. Micro-CT 2D images and 3D volume renderings of transversal and sagittal sections of *Terebellides norvegica* (ZMBN116378). (A) digestive tract; (B–C) oesophagus; (D–E) fore stomach. (A) shows position of sections (B), (C), (D) and (E). Scale bars: A–E = 1 mm.
Abbreviations: br – branchiae; ct – connective tissue; dbv – dorsal blood vessel; dg – digestive gland; dgh – digestive gland height; dgw – digestive gland width; fs – fore stomach; hs – hind stomach; ms – mid stomach; ne – nephridia; oes – oesophagus.

bears 3 lamellae (Fig. 3B and 7D). Fore stomach continues in a short mid stomach to which the digestive gland is connected. Mid stomach followed by hind stomach (length: 1687 and 967.6 μm; wall thickness: 97 and 63.5 μm) is about as long as fore + mid stomach (Supp. Figs. 4A and D). Fore intestine is 90.5 μm long and wall thickness is of 89 μm (Supp. Fig. 4E).

3.1.5. *Terebellides kirkegaardi* Parapar, Martin & Moreira, 2020 (Fig. 7A–D; Supp. Figs. 5A–E; Supp. Table 1)

Material examined: 2 specimens. NHMD1235438: 29 mm long, 1.79 mm wide; NHMD1235439: 23 mm long, 1.7 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity is filled by the digestive tract in posterior region of the body. Nephridia wall thickness ranges from 47 to 50 μm (Supp. Figs. 5A and B). Mouth opens into oesophagus (Supp. Fig. 5B) which leads to fore stomach (length: 642 and 1050 μm, respectively; wall thickness: 130 and 96 μm); the latter is surrounded ventrally by a voluminous digestive gland (length: 1087 and 1409 μm; width: 890 and 623.9 μm; height: 603 and 659.9 μm) (Fig. 7B and C; Supp. Fig. 5C) that bears 5 lamellae (Fig. 7D). Fore stomach continues in a short mid stomach to which the digestive gland is connected. Mid stomach followed by hind stomach (length: 1362 and 1919 μm; wall thickness: 85.3 and 68.9 μm) is about twice the length of fore + mid stomach (Supp. Figs. 5A and D). Fore intestine could not be measured due to the large amount of sediment within (Supp. Fig. 5E).

3.1.6. *Terebellides lavesquei* Barroso, Moreira, Capa, Nygren & Parapar, 2022 (Figs 2C, 3C, 7A–D; Supp. Figs. 6A–E; Supp. Table 1)

Material examined: 2 specimens. ZMBN116321: 30 mm long, 1.36 mm wide; ZMBN116326: 8 mm long, 1.13 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity in the thoracic region is totally filled by a pair of nephridia and by the digestive tract in the rest of the body. Nephridia wall thickness ranges from 162 to 171.2 μm (Fig. 2C; Supp. Figs. 6A and B). Mouth opens into oesophagus which leads to fore stomach (Supp. Fig. 6B) (length: 2011 and 1983.7 μm, respectively; wall thickness: 122 and 90.6 μm); the latter is surrounded ventrally by a voluminous digestive gland (length: 1757 and 1893 μm; width: 1172 and 1369.4 μm; height: 1158 and 1087.4 μm) (Fig. 7B and C; Supp. Fig. 6C) that bears 6 lamellae (Fig. 3C and 7D). Fore stomach continues in a short mid stomach to which the digestive gland is connected. Mid stomach followed by hind stomach (length: 2194 and 1671 μm; wall

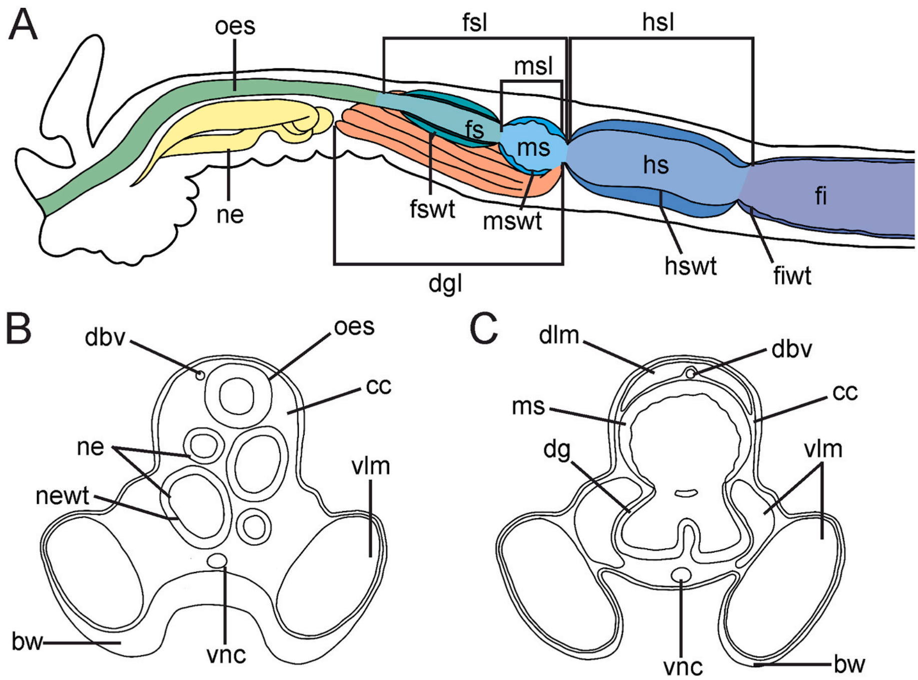

Fig. 5. Schematic drawings of transversal and sagittal sections of *Terebellides williamsae* (ZMBN116246). (A) digestive tract; (B) oesophagus and nephridia; (C) mid stomach and digestive gland. Abbreviations: bw – body wall; cc – coelomic cavity; dbv – dorsal blood vessel; dg – digestive gland; dgl – digestive gland length; dlm – dorsal longitudinal musculature; fi – fore intestine; fiwt – fore intestine wall thickness; fs – fore stomach; fsl – fore stomach length; fswt – fore stomach wall thickness; hs – hind stomach; hsl – hind stomach length; hswt – hind stomach wall thickness; ms – mid stomach; msl – mid stomach length; mswt – mid stomach wall thickness; ne – nephridia; newt – nephridia wall thickness; oes – oesophagus; vlm – ventral longitudinal musculature; vnc – ventral nerve cord.

thickness: 111 and 100.7 µm) is about as long as fore + mid stomach (Supp. Figs. 6A and D). Fore intestine could not be measured due to the large amount of sediment within (Supp. Fig. 6E).

3.1.7. *Terebellides nkossa* Parapar, Martin & Moreira, 2020 (Fig. 2D, 3D, 7A–D; Supp. Figs. 7A–E; Supp. Table 1)

Material examined: 2 specimens. MNCN16.01/90300: 12 mm long, 0.52 mm wide; MNCN16.01/90301: 11 mm long, 0.52 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity is filled by the digestive tract in the posterior region of the body. Nephridia wall thickness ranges from 12 to 27 µm (Fig. 2D; Supp. Figs. 7A and B). Mouth opens into oesophagus (Supp. Fig. 7B) which leads to fore stomach (length: 706 and 523.4 µm, respectively; wall thickness: 29.7 and 35.8 µm); the latter is surrounded ventrally by a voluminous digestive gland (length: 765 and 566.5 µm; width: 409 and 466.1 µm; height: 492 and 401.6 µm) (Fig. 7B and C; Supp. Fig. 7C) that bears 5 lamellae (Fig. 3D and 7D). Fore stomach continues in a short mid stomach to which the digestive gland is connected. Mid stomach followed by hind stomach (length: 765 and 674 µm; wall thickness 29.73 and 35.7 µm) is about as long as fore + mid stomach (Supp. Figs. 7A and D). Fore intestine could not be measured due to the large amount of sediment within (Supp. Fig. 7E).

3.1.8. *Terebellides norvegica* Parapar, Capa, Nygren & Moreira, 2020 (Fig. 2E, 3E and 4, 7A–D; Supp. Figs. 8A–E; Supp. Table 1)

Material examined: 3 specimens. GNM15133: incomplete specimen, 2.36 mm wide; ZMBN116378: 33 mm long, 3.42 mm wide; ZMBN116380: 20 mm long, 1.18 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity in the thoracic region is totally filled by a pair nephridia and by the digestive tract in the rest of the body. Nephridia wall thickness ranges from 49 to 176.5 µm (Fig. 2E, 4A–C). Mouth opens into oesophagus (Fig. 4A–C) which leads to fore stomach (length: 3100, 1995 and 746 µm, respectively; wall thickness: 286, 366.5 and 165 µm); the latter is surrounded ventrally by a voluminous digestive gland (length: 2837, 2827.7 and 1449 µm; width: 2584.5, 3109 and 1441 µm; height: 2617, 2077 and 1165.4 µm) (Fig. 4D and E, 7B, C; Supp. Fig. 3C) that bears 6 lamellae (Fig. 3E and 7D). Fore stomach continues in a short mid stomach (length: 1249 and 655 µm; wall thickness: 55, 108.6 and 78.7 µm) to which the digestive gland is connected (Fig. 4A, Supp. Fig. 8B). Mid stomach followed by hind stomach (length: 3178, 3176.7 and 1228 µm; wall thickness: 202.95, 271.21 and 104 µm) is about as long as fore + mid stomach (Supp. Figs. 8A and B). Fore intestine wall thickness is 176, 113 and 69 µm, respectively (Supp. Figs. 8A, D, E).

3.1.9. *Terebellides shetlandica* Parapar, Moreira & O'Reilly, 2016 (Fig. 2F, 3F, 7A–D; Supp. Figs. 9A–E; Supp. Table 1)

Material examined: 3 specimens. NMS.Z2013.07.01: incomplete specimen, 0.49 mm wide; ZMBN116221: 14 mm long, 0.76 mm wide; ZMBN116227: 13 mm long, 0.45 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity in the thoracic region is totally filled by a pair of nephridia and by the digestive tract in the rest of the body. Nephridia wall thickness ranges from 10 to 97 µm (Fig. 2F; Supp. Figs. 9A and B). Mouth opens into oesophagus (Supp. Fig. 9B) which leads to fore stomach (length: 710, 811 and 740.6 µm, respectively; wall thickness: 56.7, 98.6 and 89.5 µm); the latter is surrounded ventrally by a voluminous digestive gland (length: 920, 963.2 and 1001 µm; width: 562, 849.45 and 1131.2 µm; height: 385, 568.8 and 813.8 µm) (Fig. 7B and C; Supp. Fig. 9C) that bears 5 lamellae (Fig. 3F and 7D). Fore stomach continues in a short mid stomach to which the digestive gland is connected. Mid stomach followed by hind stomach (length: 1400, 1714.1 and 1188 µm; wall thickness: 44, 94 and 48.93 µm) is about twice as long as fore + mid stomach (Supp. Figs. 9A and D). Fore intestine wall thickness is 28.5, 106 and 61 µm, respectively (Supp. Fig. 9E).

3.1.10. *Terebellides stroemii* Sars, 1835 (Fig. 7A–D; Supp. Figs. 10A–E; Supp. Table 1)

Material examined: 1 specimen. NHMOC5898: incomplete specimen, 3.16 mm wide (Supp. Table 1).

Internal anatomy: Body cavity in the thoracic region is totally filled by a pair of nephridia and by the digestive tract in the rest of the body. Nephridia wall thickness is 60 µm (Supp. Figs. 10A and B). Mouth opens into oesophagus (Supp. Fig. 10B) which leads to fore stomach (wall thickness: 2128 µm; length 142.6 µm); the latter is surrounded ventrally by a voluminous digestive gland (length: 2080 µm; width: 1590 µm; height: 1283 µm) (Fig. 7B and C; Supp. Fig. 10C) that bears 6 lamellae (Fig. 7D). Fore stomach continues in a short mid stomach to which the

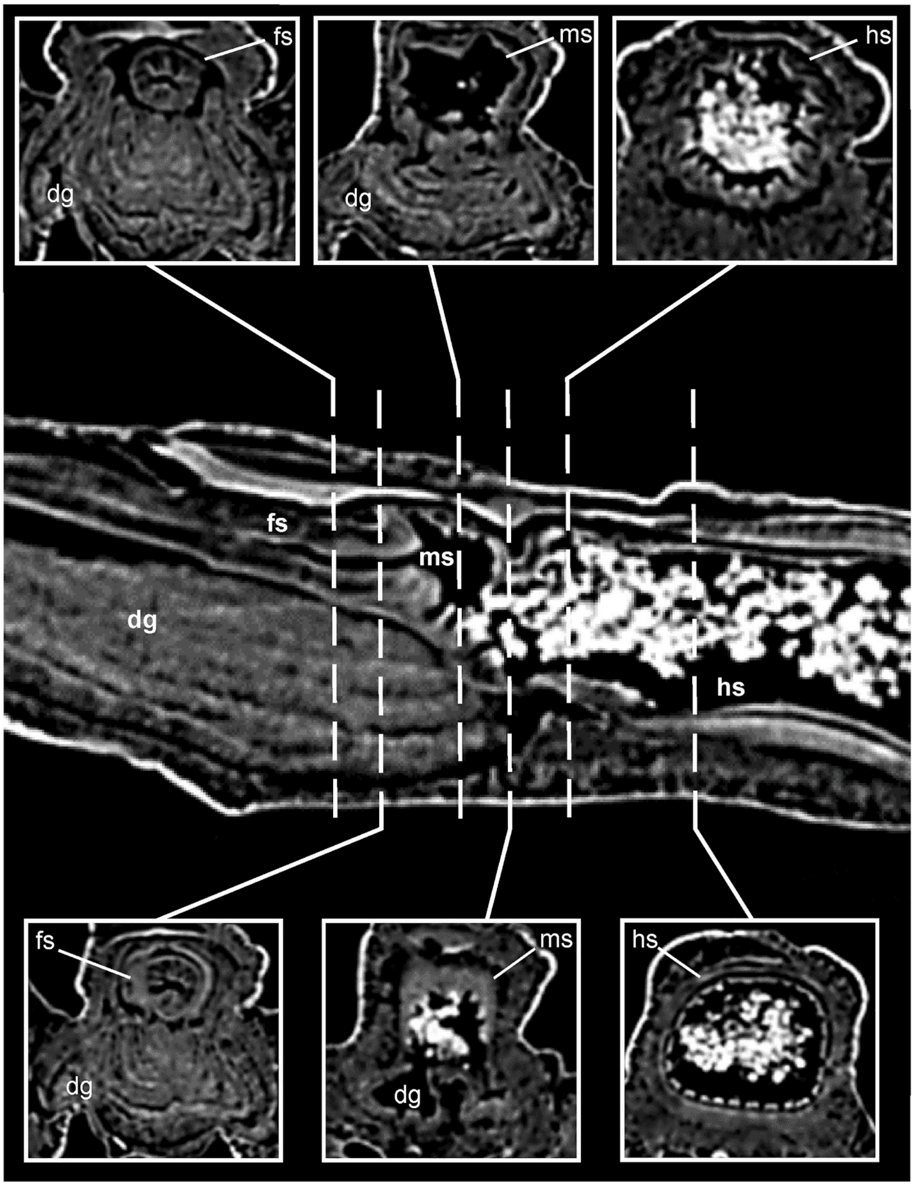

Fig. 6. Micro-CT 2D images of digestive tract of *Terebellides williamsae* (ZMBN116246) showing transversal sections at different regions. Abbreviations: dg – digestive gland; fs – fore stomach; hs – hind stomach; hsl – hind stomach length; ms – mid stomach.

digestive gland is connected. Mid stomach followed by hind stomach (length: 2515 μm; wall thickness: 156.5 μm) is about as long as fore + mid stomach (Supp. Figs. 9A, D, E). Fore intestine wall thickness is 166.5 μm (Supp. Fig. 11A).

3.1.11. Terebellides williamsae Jirkov, 1989 (Fig. 5A–C, 6, 7A–D; Supp. Figs. 11A–E; Supp. Table 1)

Material examined: 3 specimens. GNM15108: 30 mm long, 1.59 mm wide; ZMBN116246: 14 mm long, 0.65 mm wide; GNM15109: 21 mm long, 0.81 mm wide (Fig. 7A; Supp. Table 1).

Internal anatomy: Body cavity in the thoracic region totally occupied by a pair of nephridia and by the digestive tract in the rest of the body. Nephridia wall thickness ranges from 65 to 186.2 μm (Fig. 5B; Supp. Figs. 11A and B). Mouth opens into oesophagus (Fig. 5A; Supp. Fig. 11B) which leads to fore stomach (length: 1307, 1051 and 1521 μm, respectively; wall thickness: 303, 113.3 and 74 μm); the latter is surrounded ventrally by a voluminous digestive gland (length: 2248.3, 1430.5 and 1966.6 μm; width: 1379, 322 and 705 μm; height: 1048, 393 and 626 μm) (Fig. 7B and C; Supp. Fig. 11C) that bears 3 lamellae (Fig. 7D). Fore stomach continues in a short mid stomach (length: 541, 202 and 208 μm; wall thickness: 82.7, 35.76 and 39.4 μm) to which the digestive gland (Fig. 5C and 6) is connected (Fig. 5A and 6; Supp. Fig. 11A). Mid stomach followed by hind stomach (length: 1750, 1089 and 1710 μm; wall thickness: 117, 53.3 and 60.6 μm) is about as long as fore + mid stomach (Supp. Fig. 11D). Fore intestine wall thickness is 87.8, 31.6 and 70.6 μm, respectively (Supp. Fig. 11E).

3.2. Discriminant characters

Examination of specimens revealed three characters that show interspecific differences: 1) the relative length of hind stomach compared to that of fore + mid stomach combined, 2) the size of nephridia and wall thickness, and 3) the number of lamellae of the digestive gland.

1) **Length of stomach regions**

Two morphotypes were distinguished regarding relative length of

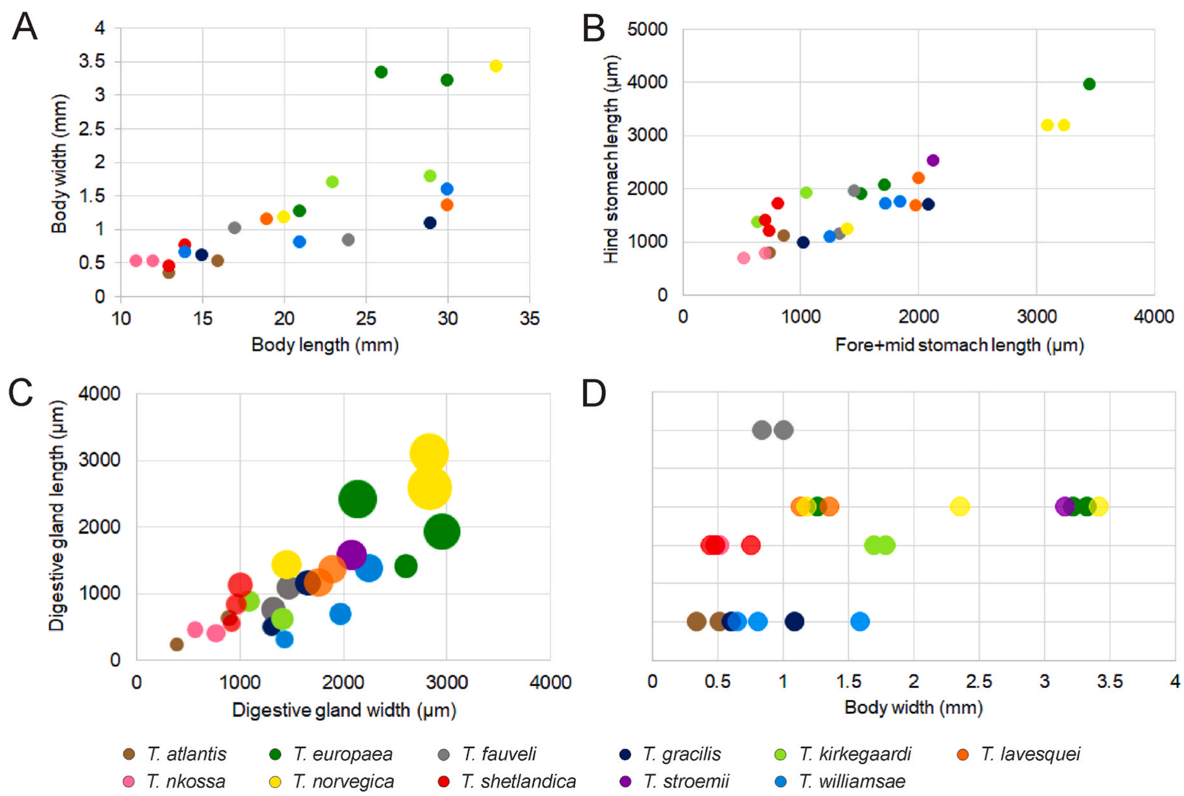

Fig. 7. Relationship between several body measurements in the *Terebellides* species studied. (A) body length *vs.* body width; (B) fore stomach length *vs.* hind stomach length; (C) digestive gland length *vs.* digestive gland width (relative digestive gland height also shown and represented proportionally according to circle size); (D) number of digestive gland lamellae *vs.* body width.

stomach regions (Fig. 5A and 7B).

Type 1:1 - hind stomach is similar in length to fore + mid stomach combined. Present in *T. atlantis*, *T. europaea*, *T. gracilis*, *T. lavesquei*, *T. norvegica*, *T. stroemii*, *T. williamsae* (NEA), *T. fauveli* and *T. nkossa* (GGA).

Type 1:2 - hind stomach is twice as long as fore + mid stomach. *T. shetlandica* (NEA) and *T. kirkegaardi* (GGA).

2) **Size of nephridia**

Two morphotypes were distinguished regarding nephridial size and nephridial wall thickness (Figs. 2 and 5B).

Type 1 - nephridia completely filling the anterior (thoracic) body cavity; nephridial wall thickness not uniform. Present in *T. atlantis*, *T. europaea*, *T. lavesquei*, *T. norvegica*, *T. shetlandica*, *T. stroemii*, *T. gracilis* and *T. williamsae* (NEA) (Fig. 2B, C, E, F).

Type 2 - nephridia not filling the anterior (thoracic) body cavity; nephridial wall thickness fairly uniform. *T. fauveli*, *T. nkossa* and *T. kirkegaardi* (GGA) (Fig. 2A, D).

3) **Digestive gland lamellae**

Four types were distinguished regarding number of lamellae in the digestive gland (Figs. 3 and 7D).

Type 1 - Three lamellae. Present in *T. atlantis*, *T. gracilis* and *T. williamsae* (NEA) (Fig. 3B).

Type 2 - Five lamellae. Present in *T. shetlandica* (NEA), *T. kirkegaardi* and *T. nkossa* (GGA) (Fig. 3D, F).

Type 3 - Six lamellae. Present in *T. europaea*, *T. lavesquei*, *T. norvegica* and *T. stroemii* (NEA) (Fig. 3C, E).

Type 4 - Eight lamellae. Present in *T. fauveli* (GGA) (Fig. 3A).

Each species shows always the same number of lamellae in the digestive gland regardless body size (Fig. 7D).

The eight species present in NEA waters (i.e. *T. atlantis*, *T. europaea*, *T. gracilis*, *T. lavesquei*, *T. norvegica*, *T. shetlandica*, *T. stroemii* and *T. williamsae*) are characterised by having highly developed nephridia provided with a thick wall that fill the thoracic body cavity; they differ from each other in the number of digestive gland lamellae, that ranges from 3 to 6. Furthermore, all species but *T. shetlandica* have a stomach of type 1:1. The three species present in GGA waters, namely *T. fauveli*, *T. kirkegaardi* and *T. nkossa*, are characterised by having small nephridia provided with a thin wall that do not fill the thoracic body cavity. *Terebellides kirkegaardi* and *T. nkossa* have five lamellae in the digestive gland while *T. fauveli* bears 8; the stomach of *T. fauveli* and *T. nkossa* is of type 1:1 while in *T. kirkegaardi* corresponds to type 1:2.

Furthermore, examination of the general internal anatomy of the eleven studied species of *Terebellides* suggests the presence of interspecific differences regarding three characters (see above). However, those seem not related to the known bathymetry or geographic distribution of each species apart from that referred to the size of nephridia. Thus, sizes differ between the NEA and GGA studied species: the nephridia are big and fill the thoracic body cavity in NEA species while in GGA species are much shorter, not filling the body cavity (Supp. Table 1).

4. Discussion

The relevance and the potential of micro-CT in the study of the anatomy of invertebrates and particularly in polychaete annelids have been fully assessed in previous works (e.g. Faulwetter et al., 2013, Paterson et al., 2014, 2017, 2018a, b, 2019, Watson & Faulwetter, 2017). This technique is also an useful tool for examination of the internal anatomy of other taxa, such as gastropods (Sumner-Rooney et al., 2019), solenogastres (Martínez-Sanjuán et al., 2022), bivalves (Marcondes Machado et al., 2019), insects (Schoborg et al., 2019; Killiny & Brodersen, 2022) and echinoderms (Ziegler, 2019).

4.1. Micro-CT in the study of Terebellides anatomy

The first study of the internal anatomy of *Terebellides* using micro-CT was done by Parapar & Hutchings (2014) who described in *T. stroemii* the regionalisation of the digestive tract and particularly the stomach. Later, Parapar et al. (2016a) also reported these subdivisions in the digestive tract in *T. shetlandica* and the relative length of stomach regions as previously reported for the genus by Michel et al. (1984), Williams (1984), Penry & Jumars (1990) and Parapar & Hutchings (2014). Parapar et al. (2016b) found again the same digestive tract regionalisation in *T. persiae* and confirmed the previous observations regarding the arrangement of the circulatory, nervous and excretory systems provided by Steen (1883) and Jouin-Toulmond & Hourdez (2006).

Here, we examined the aforementioned anatomical features in eleven *Terebellides* species collected in previous studies along the eastern Atlantic Ocean, and identified several characters that seem to show interspecific variability and therefore with potential taxonomic relevance.

The digestive system has been the most studied element in the internal anatomy of *Terebellides* (e.g., Steen, 1883, Wirén, 1885, Michel et al., 1984, Penry & Jumars, 1990, Parapar & Hutchings, 2014, Parapar et al., 2016a, b). All previous work highlights the division of the stomach in distinct fore and hind regions. Nevertheless, our observations detected a third region, named here as mid stomach, which is located between the fore and hind stomach and is connected to the digestive gland. The mid stomach differs from the other regions in epithelia thickness, but it can be easily overlooked when the digestive tract is filled with sediment. Michel et al. (1984) reported that the lumen of the digestive gland lamellae enters into the digestive tract at the boundary of the fore and hind stomach; this boundary corresponds to the region here identified as the mid stomach (Fig. 4A and 5A, C, 6). We consider the mid stomach as a distinct region from the fore stomach because of: 1) the micro-CT images showed that its epithelium is different from that of the fore and hind stomach (Fig. 4E and 6) and 2) the fore stomach is surrounded by a sheath of loose muscular connective tissue (see also Wirén, 1885; Michel et al., 1984) while the mid stomach lacks such sheath (Fig. 4A; Supp. Fig. 12). The function of the mid stomach could be related to that of the digestive gland, but examination of histological sections and transmission electron microscopy studies are necessary to assess this hypothesis.

4.2. Characters with potential taxonomic value

Taxonomy of genus *Terebellides* mostly relied on external characters, mainly related to branchiae (Parapar et al., 2016a) but also to uncinal morphology as proposed by Parapar et al. (2020a, b). In this study, we tested the potential of micro-CT to search for new taxonomic characters of internal anatomy.

Regarding stomach regionalisation, Williams (1984) reported that the fore and hind stomach of *T. atlantis*, *T. californica*, *T. reishi* and *T. stroemii* were similar in length to each other (type 1:1) while *T. distincta* showed a fore stomach twice the length of hind stomach (type 2:1). Recently, Parapar & Hutchings (2014) confirmed proportions 1:1 for *T. stroemii* when designing its neotype, and Parapar et al. (2016a, b) reported the same value for *T. shetlandica* and *T. persiae*. However, we re-examined the *T. shetlandica* specimen studied by Parapar et al. (2016a) and found that this proportion is actually 1:2. This discrepancy may be due to the state of preservation of the specimen, which shows a depression between both stomach regions that was not considered in the measurements done by these authors.

We did not compare the relative length of the mid stomach against the fore and hind regions because many specimens have the digestive tract filled with sediment; therefore, accurate measurements of this region could not be properly done. In specimens with empty gut, the mid stomach was actually measured and the combined length of the fore and mid stomach was compared to that of the hind stomach and used to define the two aforementioned morphotypes of stomachs.

Previous work (i.e., Steen, 1883; Michel et al., 1984; Parapar et al., 2016b) reported a pair of nephridia in the thoracic region of *Terebellides*, but they do not mention whether nephridia differ among species. Our study showed that two types of nephridia can be defined regarding their size and wall thickness and each of them is consistently present in species from the same geographic area. i.e. type 1 is present in NEA species while GGA ones show type 2. Steen (1883) examined several specimens identified as *T. stroemii* and illustrated some structures in the oesophagus that were named as salivary glands ("speicheldrüsen"). However, they might correspond, in fact, to large nephridia of type 1, i.e., those filling the coelomic body cavity and provided with walls not showing a uniform thickness. Two years later, Wirén (1885) illustrated similar structures, this time named as segmental organs ("segmentalorgan") that we consider that should also correspond to type 1 nephridia.

The digestive gland in *Terebellides* is composed by two lobes that are loosely joined on their ventral side but closely connected dorsally defining a variable number of lamellae (Michel et al., 1984). Previous works did not explicitly mention whether this number of lamellae is or not constant for a given species or among species. For instance, Steen (1883) studied specimens identified as *T. stroemii* and provided a drawing showing a section of the digestive gland with at least five lamellae. Wirén (1885) counted instead six lamellae in other specimens also attributed to *T. stroemii* and suggests that Steen (1883) was likely wrong. Here, we observed that *T. kirkegaardi*, *T. nkossa* and *T. shetlandica* bear five lamellae while our specimen of *T. stroemii* showed six lamellae. There might be two possible explanations for these discrepancies: the drawing by Steen (1883) does not show a full view of all lamellae or, alternatively, the specimens illustrated do not actually correspond to *T. stroemii*. On the other hand, the length, width, and height of the digestive gland did not show consistent differences among species.

Eight of the eleven species studied here were included in the four groups defined by Nygren et al. (2018) in their molecular analyses of NEA *Terebellides*: *T. europaea*, *T. norvegica* and *T. stroemii* (group A), *T. atlantis*, *T. lavesquei* and *T. shetlandica* (group B), and *T. gracilis* and *T. williamsae* (group D). Group A and D species have type 1 nephridia and stomachs of type 1:1, while digestive gland has 6 lamellae in Group A and 3 in Group D. Finally, Group B has type 1 nephridia and shows a high interspecific variability in proportions of stomach regions length (1:1 and 1:2) and in the number of digestive gland lamellae (3, 5 and 6).

4.3. Advantages, disadvantages, and limitations of micro-CT

Paterson et al. (2014) highlighted several advantages of micro-CT for examination of specimens when compared to other techniques: it is relatively fast and cheap allowing for visualisation of the anatomy in 2D and 3D without deteriorating the material, which is particularly relevant when studying type specimens. Marcondes Machado et al. (2019) also pointed out that it also allows for examination of the internal body organs in their actual position. Recently, Martínez-Sanjuán et al. (2022) showed that this technique also facilitates taking accurate measurements of different elements of internal anatomy. Here, we show that the micro-CT is also useful to provide measurements of the length of stomach regions, and also to count lamellae under conditions where traditional histological techniques may fail as it happens in sedimentivorous animals (e.g. *Terebellides* species), which often have the gut filled with sediment that does not allow for a proper sectioning.

Micro-CT has also some disadvantages. For instance, Paterson et al. (2014) reported that image volume is large, and rendering can be consequently time-consuming. Likewise, there are no specific stains for body tissues in polychaetes that can improve the quality of the resulting images, which also applies for other invertebrates such as molluscs. Other disadvantages observed in this study are: 1) it is not possible to properly confirm whether the digestive tract is broken and/or adequately positioned until the specimen is scanned (cfr. Supp. Fig.

10A); 2) proper observations of some anatomical features (e.g., accurate measurements and delineation of actual boundaries of each gut region) are not possible when the specimens are filled with sediment (e.g., Supp. Figs. 3 and 5). Parapar et al. (2017) also reported that some anatomical elements of smaller size/resolution than those studied here (e.g. brain) are not well visualised in some polychaete families. In bivalves, there is a lack of resolution when reconstructing small structures (<80 μm) such as nervous ganglia (Marcondes Machado et al., 2019). In contrast, Sumner-Rooney et al. (2019) point out that micro-CT enables a sufficient visualisation of the neurological innervation and ultrastructure of the eye in small gastropods of 3–5 mm in length. On the other hand, examination of some features asks for collection of live animals to prevent the digestive tract from being twisted, broken or filled with sediment; this also requires (if possible) keeping specimens alive to allow them expelling as much sediment as possible and then being subjected to a longer fixation process.

5. Conclusions

Micro-CT examination of eleven *Terebellides* species from eastern Atlantic has allowed to distinguish a distinct region between the fore and hind stomach, named here as mid stomach. This intermediate region connects the fore stomach with the lamellae of the digestive gland, its epithelium differs from that of the fore and hind stomach and also lacks a sheath of loose muscular connective tissue. Furthermore, we propose here several characters of internal anatomy that can be used to discriminate species in the genus: 1) differences in length of stomach regions (fore + mid stomach *vs*. hind stomach), 2) the size of nephridia, and 3) the number of lamellae of the digestive gland. On the contrary, the length, width and height of the digestive gland did not show consistent differences among species. Anyway, the taxonomic validity of the characters proposed here should be tested by examination of other *Terebellides* species from different geographic areas. Nevertheless, the micro-CT has been proved again as a useful technique for taxonomic and anatomical studies that avoids irreversible damaging of specimens.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Julio Parapar reports financial support was provided by Agencia Estatal de Investigación, Ministerio de Ciencia e Innovación, Spain. Maria Barroso reports article publishing charges was provided by Universidade da Coruña, Spain.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcz.2023.06.007.

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