Connecting names:
Using a micro-CT scanner as a non-destructive method for identification of polyclads (Platyhelminthes: Polycladida)

Jorge I. Merchán Mayorga

jorgemerchandmc@gmail.com
jorgemerchanim@unimagdalena.edu.co

Marine Invertebrate Zoology
FHL 432
Summer 2025

Connecting names:
Using a micro-CT scanner as a non-destructive method for identification of polyclads (Platyhelminthes: Polycladida)

Abstract: Polyclads are free living marine flatworms that inhabit a great variety of environments, from rocky shore and coral reefs to deep water. Due to their apparent simplicity, polyclads have been mainly classified by their internal reproductive anatomy long with the eyespot's arrangement, the presence of tentacles and the pharynx type. The study of these characters is often done through histological sectioning which requires obtaining mature specimens, to correctly fixate them and go through several steps before obtaining slides of the reproductive system. This approach is not only time consuming and difficult, but it is destructive. While molecular techniques have been growing in popularity in polyclad research, the availability of them is scarce and sequences still need to be accompanied of specimens' identification. Micro-CT scanning is considered a nondestructive method to observe internal structures, which also provides the ability for 3D reconstruction of them. Micro-CT imaging has been previously used on the taxonomic studies of invertebrates, including annelids and other flatworm groups. In order to test micro-CT scanning as a tool to match specimens collected around San Juan island intertidal to known species, worms were fixed, pre-stained with Lugol or PTA and mounted in paraffin to be scanned. Internal structures were unclear for all the scans irrespective of the stain used but was generally better for those stained with PTA and embedded in paraffin. Our results show that while feasible there are several aspects that affect CT images results for polyclad identification, leaving room for improvement on the methods used for the imaging.

Keywords: Computed tomography, flatworms, staining, taxonomy.

Introduction

The order Polycladida is a group of free-living marine invertebrates that inhabit a great variety of environments, including rocky shores, coral reefs, soft bottoms and abyssal waters (Newman & Cannon, 2003; Prudhoe, 1985; Quiroga et al., 2006). Characterized by their highly ramified intestine from where they receive their name, polyclads have a 'simple' flattened body. Polyclads are hermaphroditic and their reproductive anatomy has been seldom used as characters of taxonomy importance along with their eyespot arrangement, tentacles, pharynx type and color (Hyman, 1951; Prudhoe, 1985).

The common practice to identify polyclads flatworms is through the histological sectioning of a section of the body containing the reproductive structures, this process includes fixating the animal, dehydration of the tissue, embedding it in paraffin, obtaining serial sections and staining (Newman & Cannon, 1995). However, this is a lengthy process that needs to be carried carefully in order to obtain informative slides, and as such is time consuming and destructive regarding the specimens.

This being a poorly studied group of organisms, molecular information is scarce and corresponds primarily to the 28S rDNA gene which may not have enough variation to be informative at the species level in comparison to the COI mtDNA standard for most animals (Cuadrado et al., 2024). While there is an increase in the available sequences for polyclads, this is mostly restricted to regions where there is active research on the group and covers a limited number of species and families (e.g. Oya & Kajihara, 2017, 2021; Rodríguez et al., 2021).

Microfocus X-ray computed tomography (micro-CT) is a powerful, fast tool complementary for the exploration of animal anatomy including marine invertebrates (Chapuis et al., 2024; Dinley et al., 2010; Fernández et al., 2014; Parapar et al., 2019). Micro-CT scanning has been used to study museum terrestrial flatworm specimens, and even for the description of new species (Carbayo et al., 2016; Carbayo & Lenihan, 2016; Silva & Carbayo, 2020), however its use in polyclads is restricted to the observations of the nervous system of *Comoplana pusilla* (Bock, 1924) (Ikenaga et al., 2024), and the exploration of its use for polyclad taxonomy (Oya et al., 2024). Oya et al. (2024), explored the reproductive structures of *Paraplanocera oligoglena* (Schmarda, 1859) which possess a relatively complex copulatory apparatus, including an eversible cirrus (hardened structure of the male apparatus), finding that micro-CT presents a good approach to understand reproductive system organization.

However, the presence of a big complex hard structures in polyclad reproductive structures is limited to some families and is variable between species (Faubel, 1983; Prudhoe, 1985), which could represent difficulties to obtain contrasting images through micro-CT. Most species found in the San Juan region were first described by Freeman (1933) , since then others works have added to the fauna of the region (Hyman, 1939). In order to correctly match species names to collected flatworms around San Juan island this study aim was to observe flatworm reproductive structures through micro-CT scanning, generating the first micro-CT scanning observations of the species and adding color photographs to accompany species records.

Methodology

Flatworms were hand collected from under rocks, transported in tubes full of seawater and placed on flood tables. Animals were sorted according to their external appearance into morphotypes and given a tentative ID (*Kaburakia excelsa* and *Notocomplana* cf. *acticola*). Later, specimens were anesthetized with $MgCl_2$ isotonic to seawater, coaxed into filter paper and moved to a Petri dish with frozen sea water. Posteriorly, worms were transferred to 10% formalin for 12h and preserved in 70% ethanol.

To test weather specimen tissues had enough contrast in the micro-CT imaging, one of the specimens was transferred directly from the ethanol, bagged and scanned. After the initial observation, flatworms were pre-stained with either 2.5% phosphotungstic acid (PTA) or rehydrated and then fixed with 1.25% buffered Lugol and posteriorly embedded in histological paraffin. All the scans were done with a Skyscan 1273, reconstructed with the software NRecon and rendered with the software 3D Slicer. Pixel size was between 12-30 μm.

Results

In general, it was possible to distinguish and render the volume of the flatworms outline and surface independent of the stain used. However, the PTA stain provided better contrasting images in comparison to the specimens stained with Lugol. Yet it was not possible to distinguish the specific structures that compose the reproductive system of the polyclad, and only the outline of the female and male apparatus was seen in one of the flatworms stained with PTA.

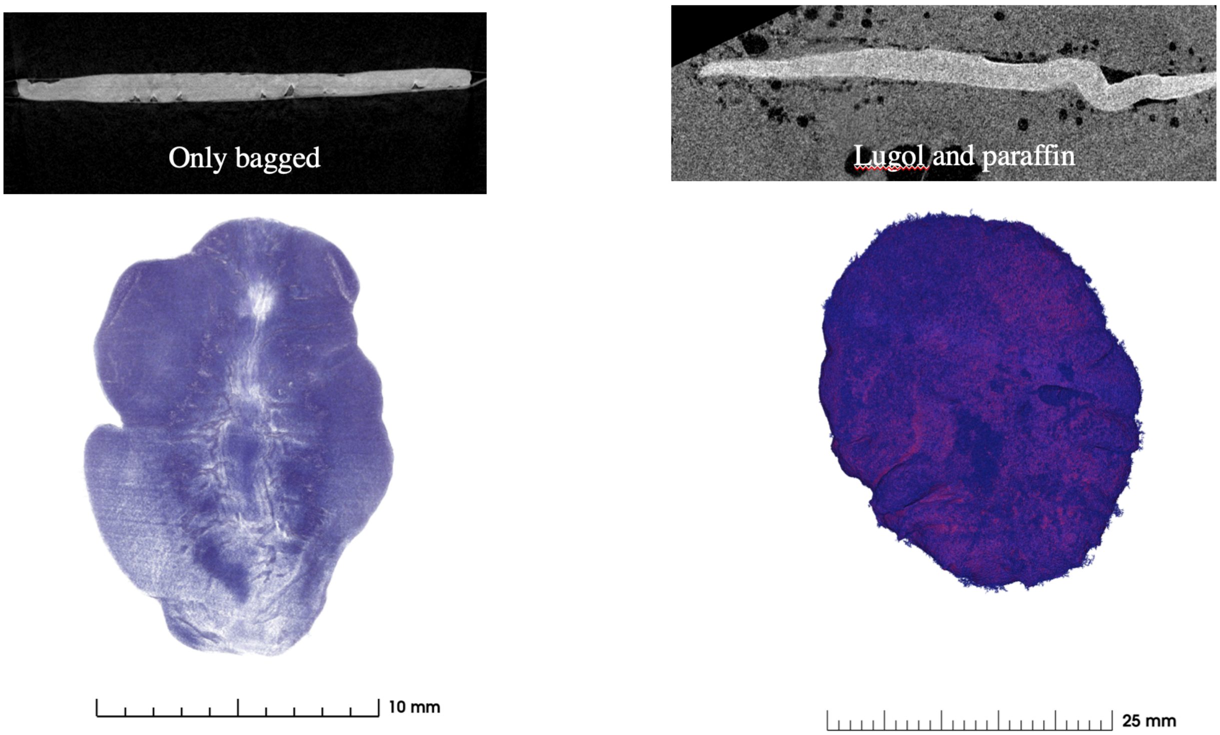


Figure 1. 3D volume rendering and 2D sagittal view of *Kaburakia excelsa* with and without Lugol stain.

PTA and Paraffin

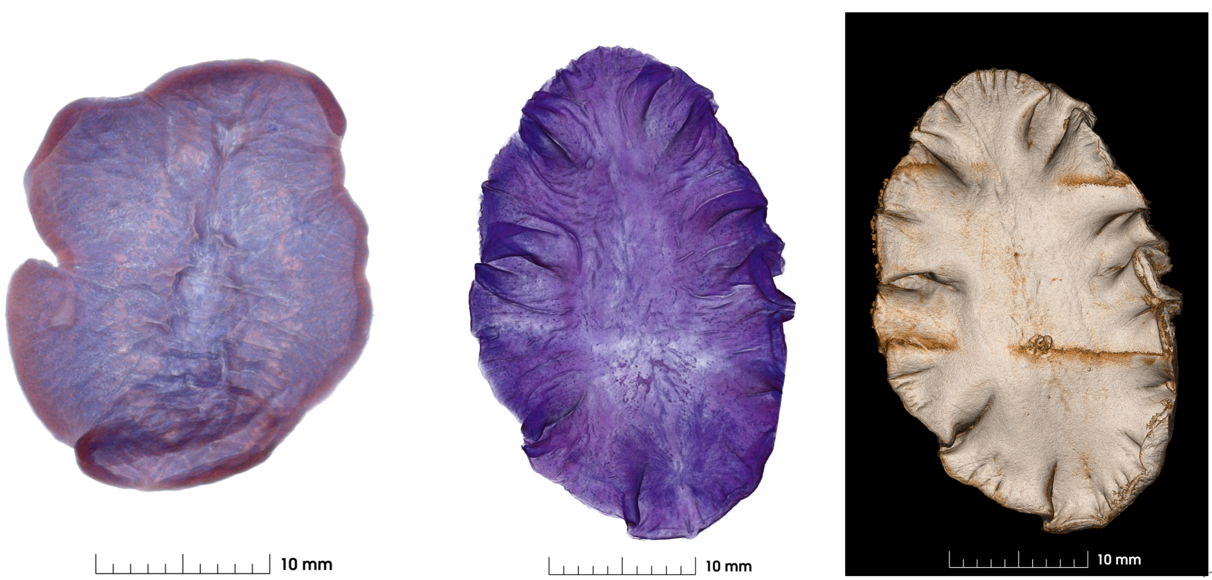
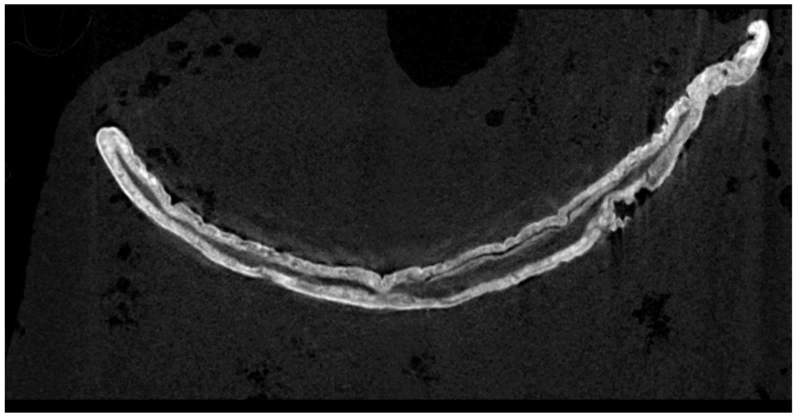


Figure 2. 3D volume rendering of *Kaburakia excelsa s*tained with PTA.



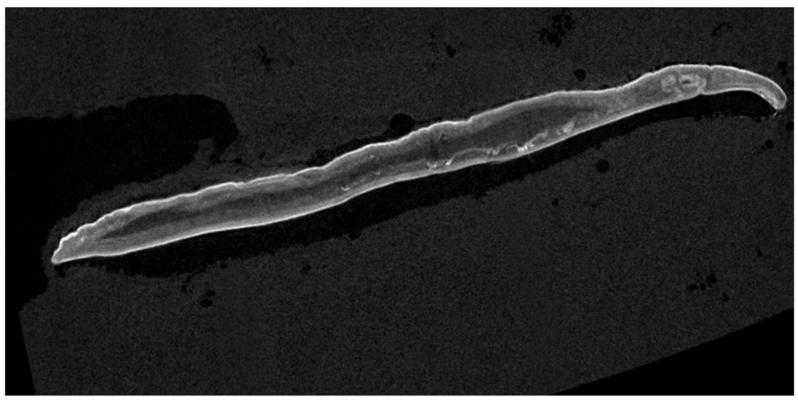


Figure 3. 2D sagittal view of *Kaburakia excelsa* with PTA stain. Left: Anterior; Right: Posterior.

Conclusion

Micro-CT scanning may represent a good option to study the internal anatomy organization of soft bodied animals such as polyclad flatworms, however the usefulness of this technic depends on the stain used for enhanced contrast of internal structures as well as the obtainable resolution from the scanner. Paraffin proved to be a useful media to obtain clear images of the external appearance of the worms and its density made it easier to separate from the organism of interest.

Bibliography

Carbayo, F., Francoy, T. M., & Giribet, G. (2016). Non-destructive imaging to describe a new species of *Obama* land planarian (Platyhelminthes, Tricladida). *Zoologica Scripta*, *45*(5), 566–578. https://doi.org/10.1111/zsc.12175

Carbayo, F., & Lenihan, J. W. (2016). Micro-computed tomography scan and virtual histological slide data for the land planarian Obama otavioi (Platyhelminthes). *GigaScience*, *5*(1), s13742-016-0119–4. https://doi.org/10.1186/s13742-016-0119-4

Chapuis, L., Andres, C.-S., Gerneke, D. A., & Radford, C. A. (2024). Bioimaging marine crustacean brain: Quantitative comparison of micro-CT preparations in an Alpheid snapping shrimp. *Frontiers in Neuroscience*, *18*. https://doi.org/10.3389/fnins.2024.1428825

Cuadrado, D., Rodríguez, J., Machordom, A., Noreña, C., Fernández-Álvarez, F. Á., Hutchings, P., & Williamson, J. (2024). Base-substitution rates of nuclear and mitochondrial genes for polyclad flatworms. *Zoosystematics and Evolution*, *100*, 863–876. https://doi.org/10.3897/zse.100.119945

Dinley, J., Hawkins, L., Paterson, G., Ball, A. D., Sinclair, I., Sinnett-Jones, P., & Lanham, S. (2010). Micro-computed X-ray tomography: A new non-destructive method of assessing sectional, fly-through and 3D imaging of a soft-bodied marine worm. *Journal of Microscopy*, *238*(2), 123–133. https://doi.org/10.1111/j.1365-2818.2009.03335.x

Faubel, A. (1983). The Polycladida, Turbellaria. Proposal and establishment of a new system. Part I. The Acotylea. *Mitt Hamb Zool Mus Inst*, *80*, 17–121.

Fernández, R., Kvist, S., Lenihan, J., Giribet, G., & Ziegler, A. (2014). Sine Systemate Chaos? A Versatile Tool for Earthworm Taxonomy: Non-Destructive Imaging of Freshly Fixed and Museum Specimens Using Micro-Computed Tomography. *PLOS ONE*, *9*(5), e96617. https://doi.org/10.1371/journal.pone.0096617

Freeman, D. (1933). The Polyclads of the San Juan Region of Puget Sound. *Transactions of the American Microscopical Society*, *52*(2), 107. https://doi.org/10.2307/3222188

Hyman, L. H. (1939). *New species of flatworms from North, Central, and South America*. *86*(3055).

Hyman, L. H. (1951). *The invertebrates: Platyhelminthes and Rhynchocoela, the acoelomate Bilateria*. (Vol. 2). McGraw-Hill Book Company. https://www.cabdirect.org/cabdirect/abstract/19510800619

Ikenaga, T., Kobayashi, A., Takeuchi, A., Uesugi, K., Maezawa, T., Shibata, N., Sakamoto, T., & Sakamoto, H. (2024). Volume X-Ray Micro-Computed Tomography Analysis of the Early Cephalized Central Nervous System in a Marine Flatworm, Stylochoplana pusilla. *Zoological Science*, *41*(3). https://doi.org/10.2108/zs230082

Newman, L. J., & Cannon, L. (2003). *Marine Flatworms: The World of Polyclads*. Csiro Publishing. https://doi.org/10.1071/9780643101197

Newman, L. J., & Cannon, L. R. (1995). The importance of the fixation of colour, pattern and form in tropical Pseudocerotidae (Platyhelminthes, Polycladida). *Hydrobiologia*, *305*, 141–143.

Oya, Y., & Kajihara, H. (2017). Description of a new Notocomplana species (Platyhelminthes: Acotylea), new combination and new records of Polycladida from the northeastern Sea of Japan, with a comparison of two different barcoding markers. *Zootaxa*, *4282*(3). https://doi.org/10.11646/zootaxa.4282.3.6

Oya, Y., & Kajihara, H. (2021). Description and phylogenetic relationships of a new genus of Planoceridae (Polycladida, Acotylea) from Shimoda, Japan. *Journal of the Marine Biological Association of the United Kingdom*, *101*(1), 81–88. https://doi.org/10.1017/S0025315421000060

Oya, Y., Maeno, A., Tsuyuki, A., Kohtsuka, H., & Kajihara, H. (2024). Microfocus X-Ray Computed Tomography of Paraplanocera oligoglena (Platyhelminthes: Polycladida) with an Evaluation of Histological Sections After Scanning. *Zoological Science*, *41*(5). https://doi.org/10.2108/zs240015

Parapar, J., Caramelo, C., Candás, M., Cunha-Veira, X., & Moreira, J. (2019). An integrative approach to the anatomy of Syllis gracilis Grube, 1840 (Annelida) using micro-computed X-ray tomography. *PeerJ*, *7*, e7251. https://doi.org/10.7717/peerj.7251

Prudhoe, S. (1985). *A Monograph on Polyclad Turbellaria*. British Museum (Natural History).

Quiroga, S. Y., Bolaños, D., & Litvaitis, M. K. (2006). First Description Of Deep-Sea Polyclad Flatworms From The North Pacific: Anocellidus N. Gen. Profundus N. Sp. (Anocellidae, N. Fam.) And Oligocladus Voightae N. Sp. (Euryleptidae). *Zootaxa*. https://doi.org/10.5281/zenodo.173981

Rodríguez, J., Hutchings, P. A., & Williamson, J. E. (2021). Biodiversity of intertidal marine flatworms (Polycladida, Platyhelminthes) in southeastern Australia. *Zootaxa*, *5024*(1), 1–63. https://doi.org/10.11646/zootaxa.5024.1.1

Silva, M. S., & Carbayo, F. (2020). X-ray microcomputed tomography applied to the taxonomic study of rare material: Redescriptions of seven of Schirch's Brazilian species of land planarians (Geoplanidae, Platyhelminthes). *ZooKeys*, *910*, 1–42. https://doi.org/10.3897/zookeys.910.39486

Connecting Names:
Using a micro-CT scanner as a non-destructive method for identification of polyclads (Platyhelminthes: Polycladida)

Jorge I. Merchán Mayorga*

Marine Invertebrate Zoology
FHL 432
July 17, 2025

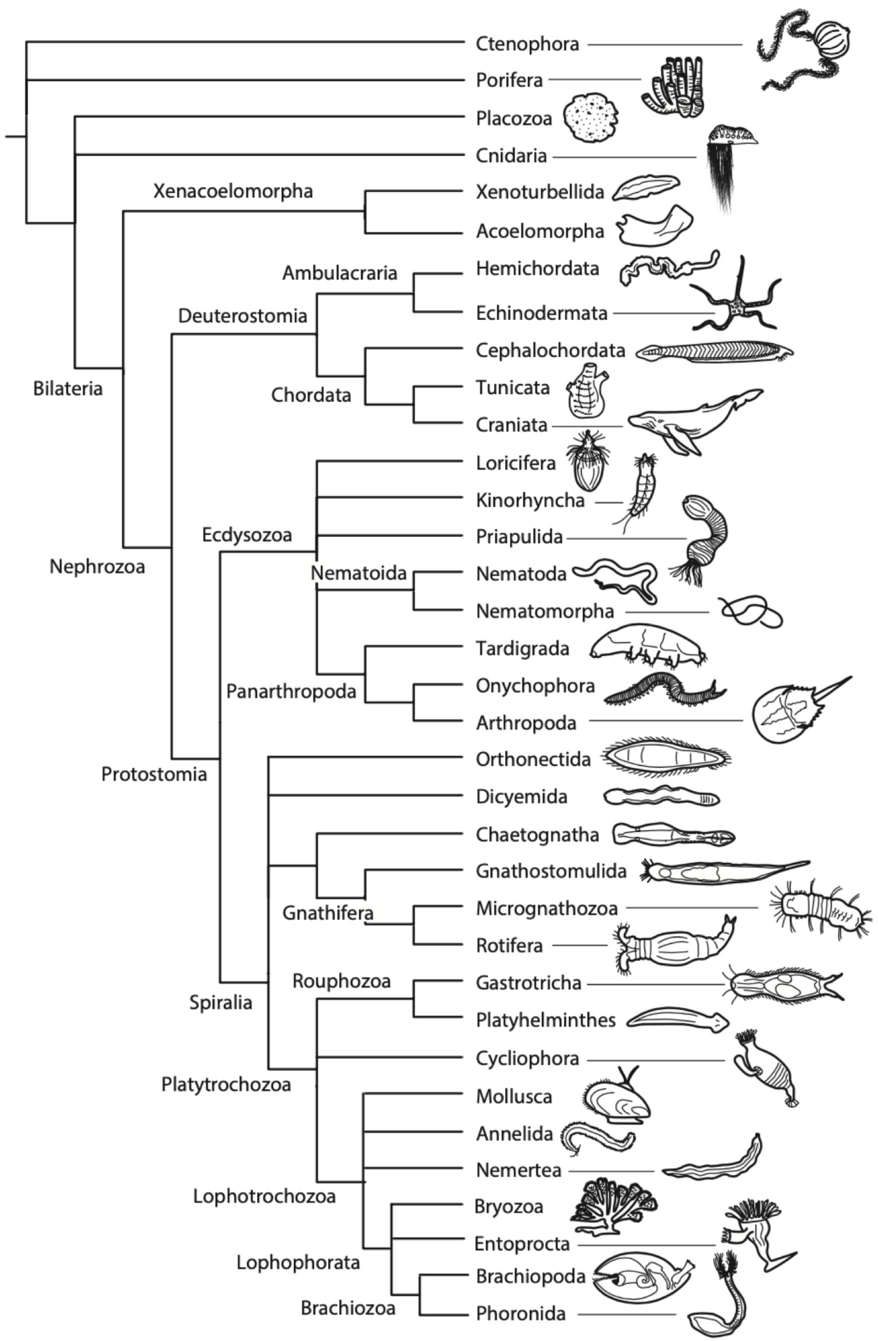
* jorgemerchandmc@gmail.com
jorgemerchanim@unimagdalena.edu.co

Connecting Names:
Using a micro-CT scanner as a non-destructive method for identification of polyclads (Platyhelminthes: Polycladida)

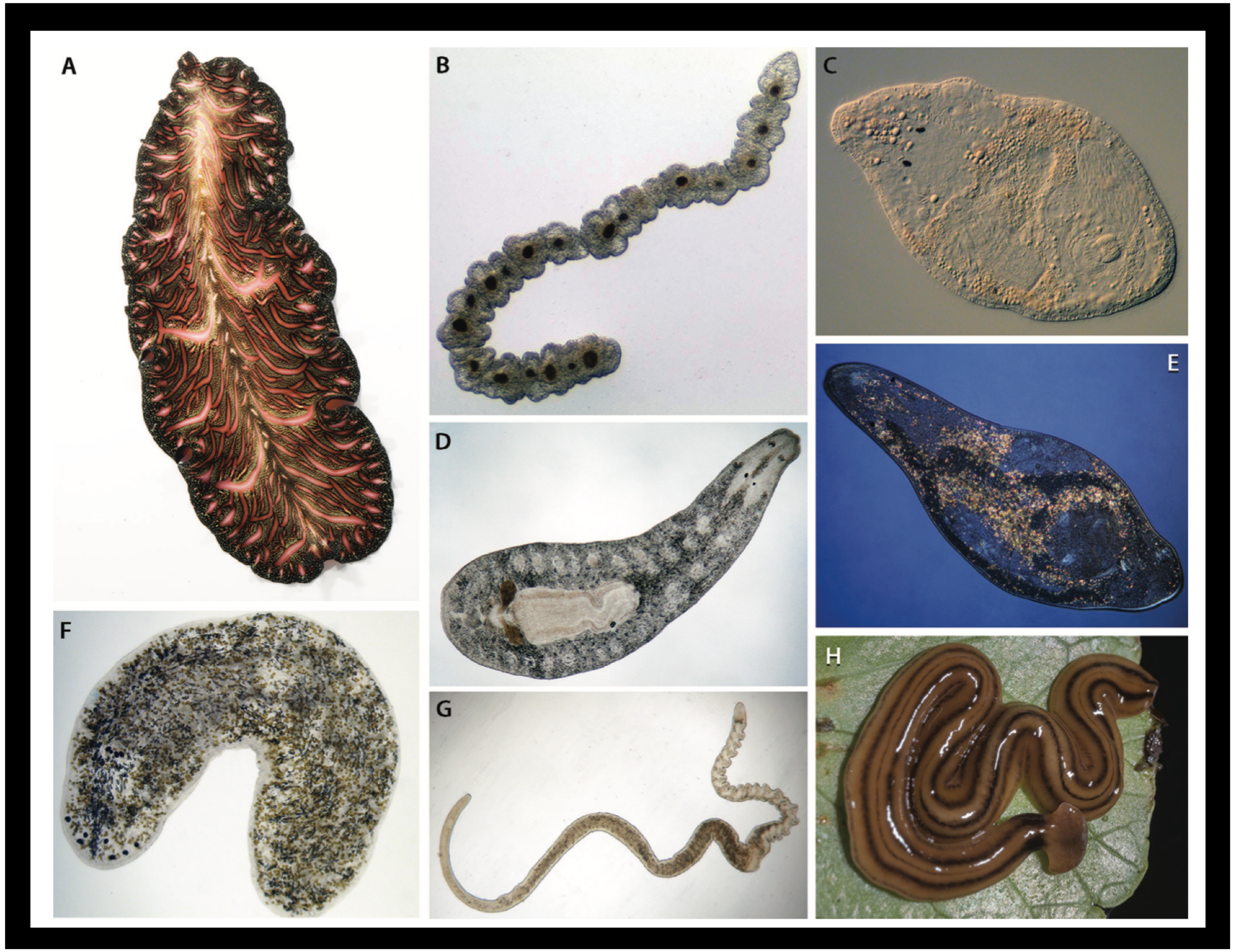
Jorge I. Merchán Mayorga*

TDLR: It works, but it could be better!

Platyhelminthes



The invertebrate tree of life (Giribet & Edgcome, 2020)

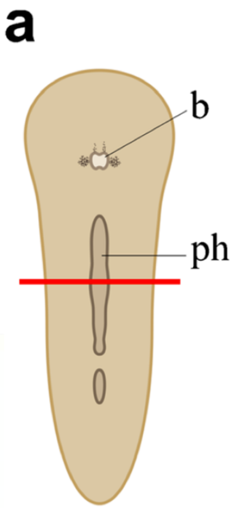


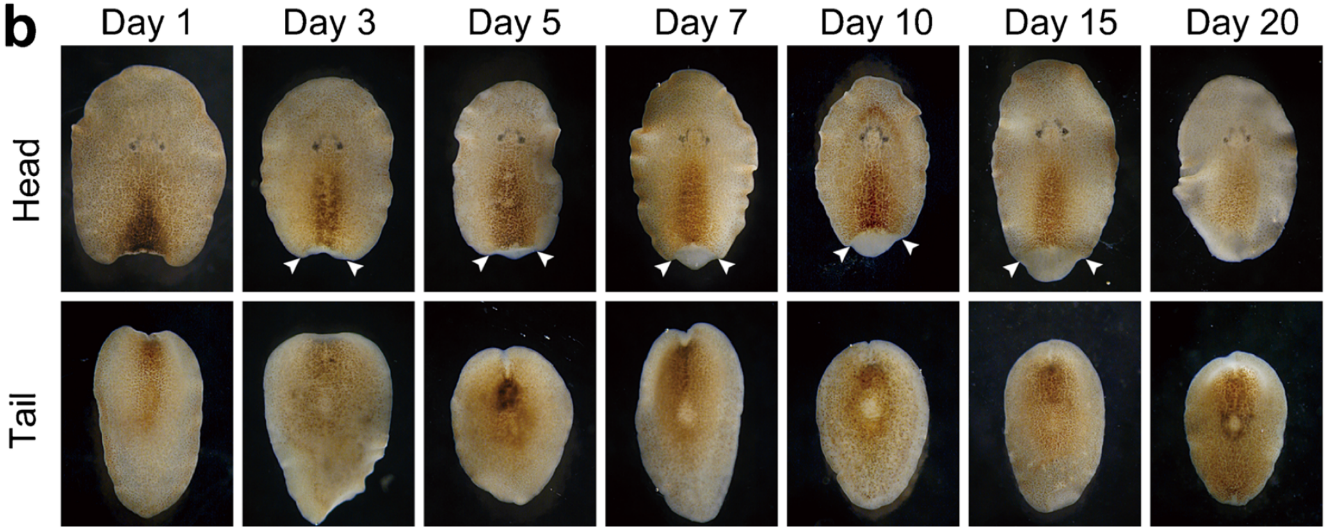
~30,000 spp.

- We only know between 8 to 32% of the subphylum Rhabditophora (Appeltans *et al*., 2012).

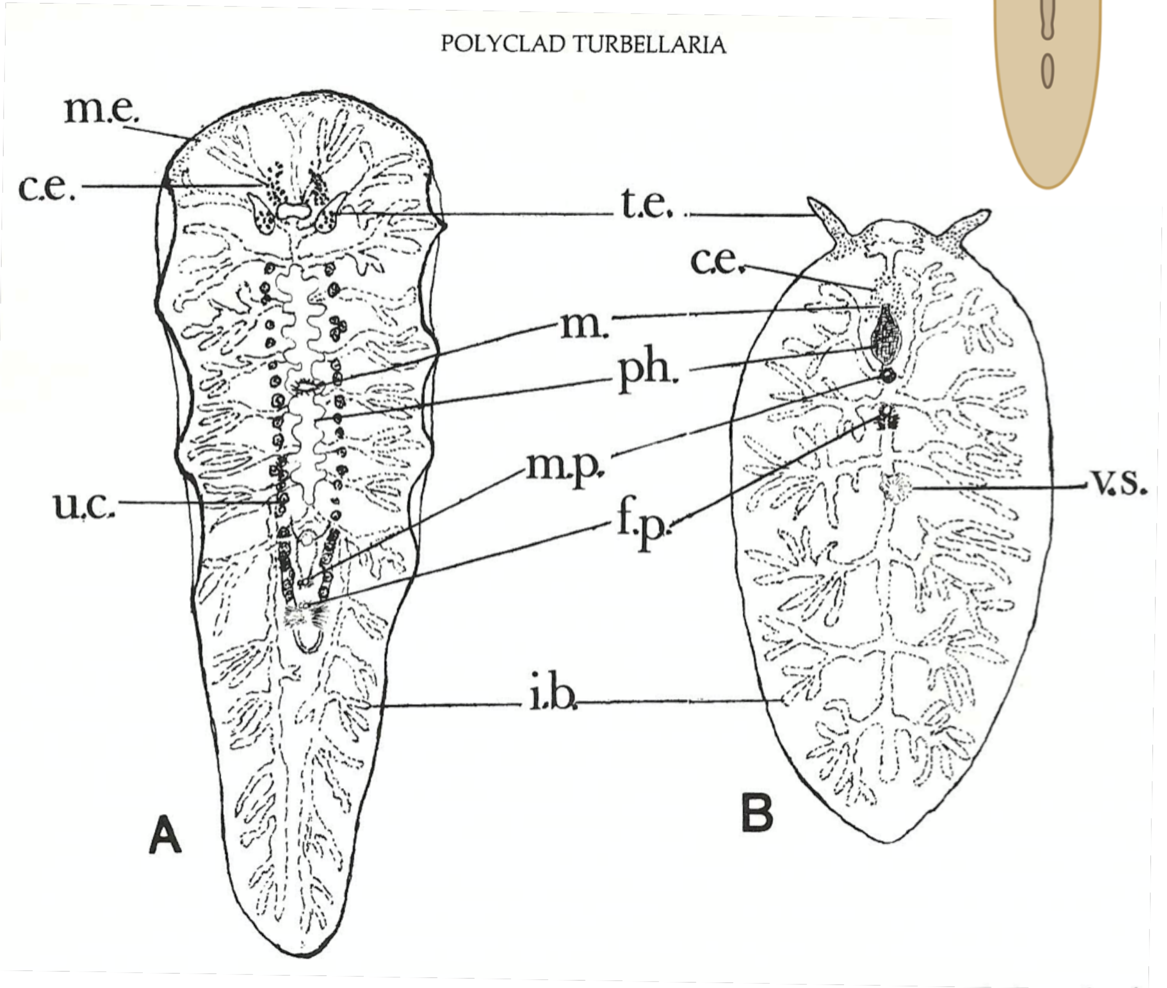
Orden Polycladida

~800 spp.





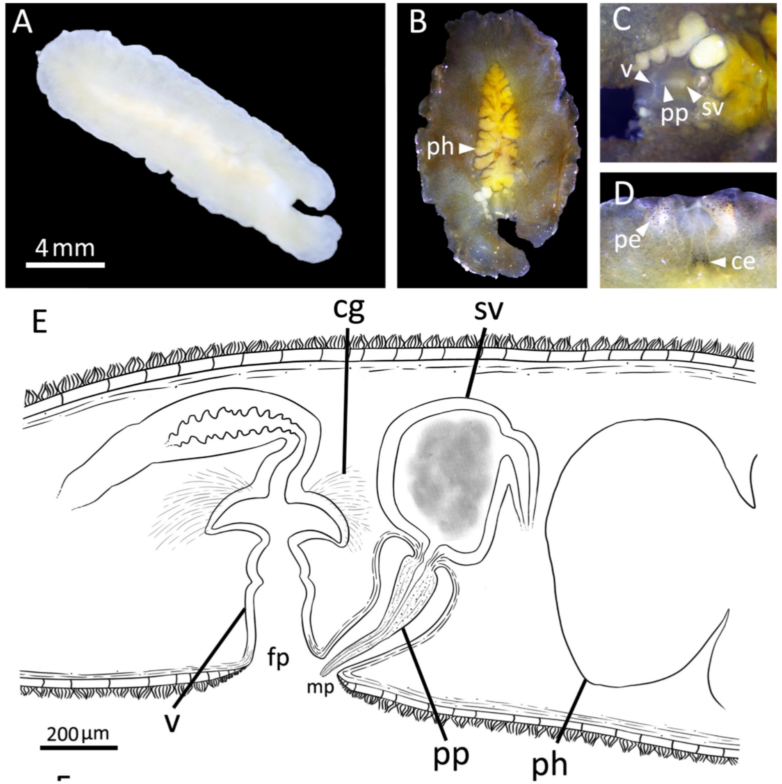
(Okano *et al*., 2015)



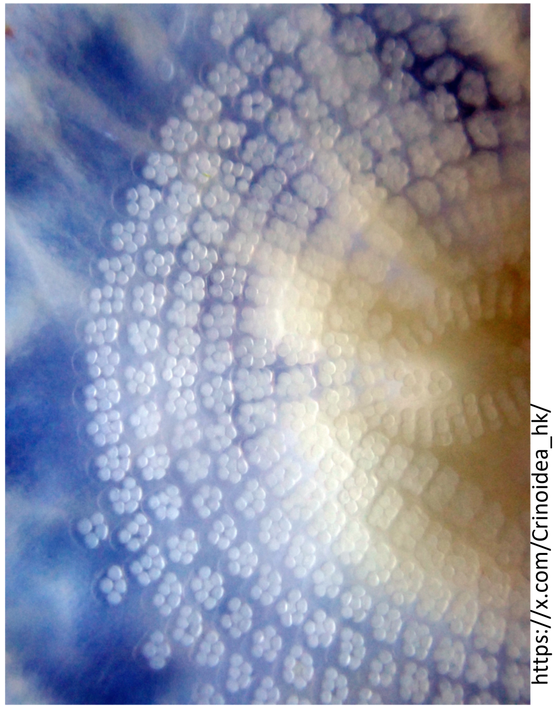
~450

~350

(Prudhoe, 1985; WORMS, 2023)



(Rodríguez *et al*., 2023)



https://x.com/Crinoidea_hk/

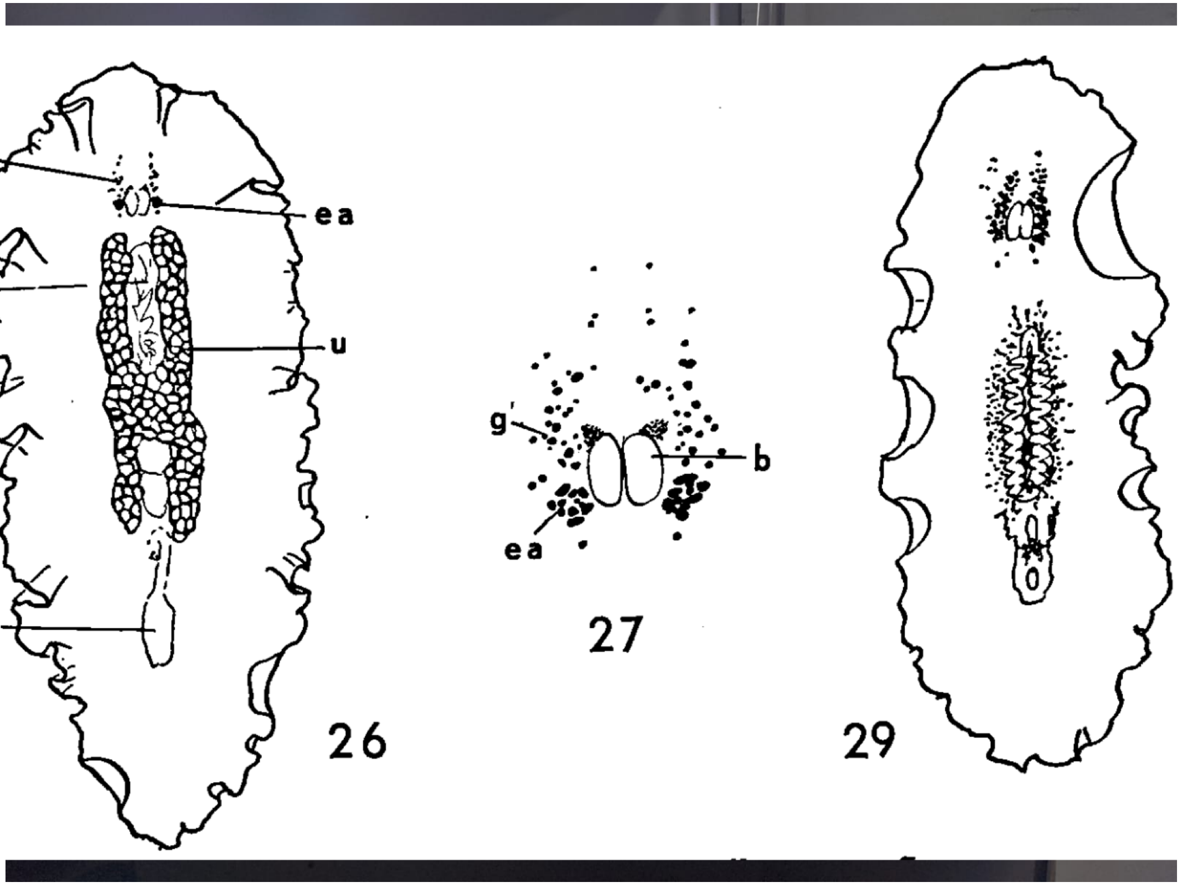
How do you tell which flatworm you are looking at?

- Color is not that informative
- The only structures that pouk out *sometimes* are tentacles
- DNA library is not big enough to match most specimens

So, what about internal organs?

‘Traditional’ taxonomy

So, it’s a lot of observation



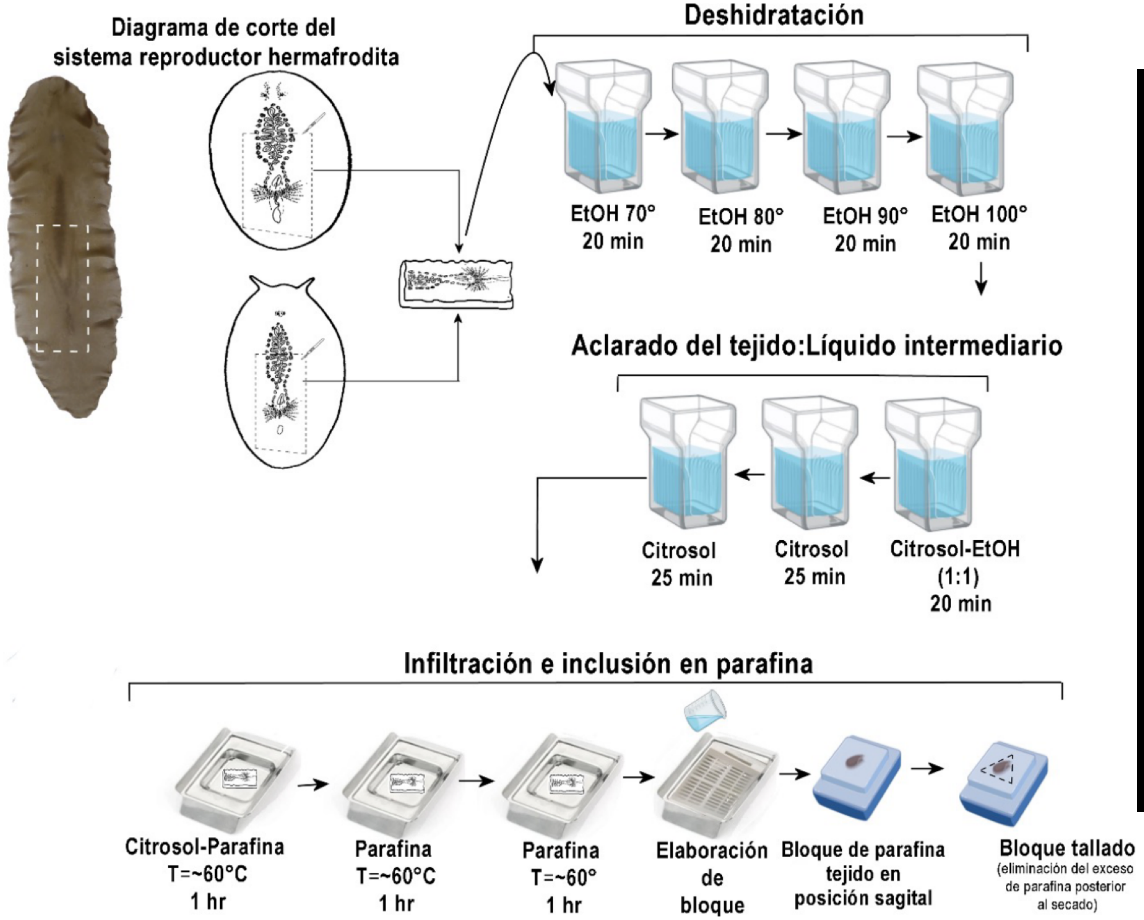
Capture and observe the worm alive!
Then you can proceed to fixation

~7.5% $MgCl_2$ – 10% Formalin

Internal morphology

So, it's complex matter

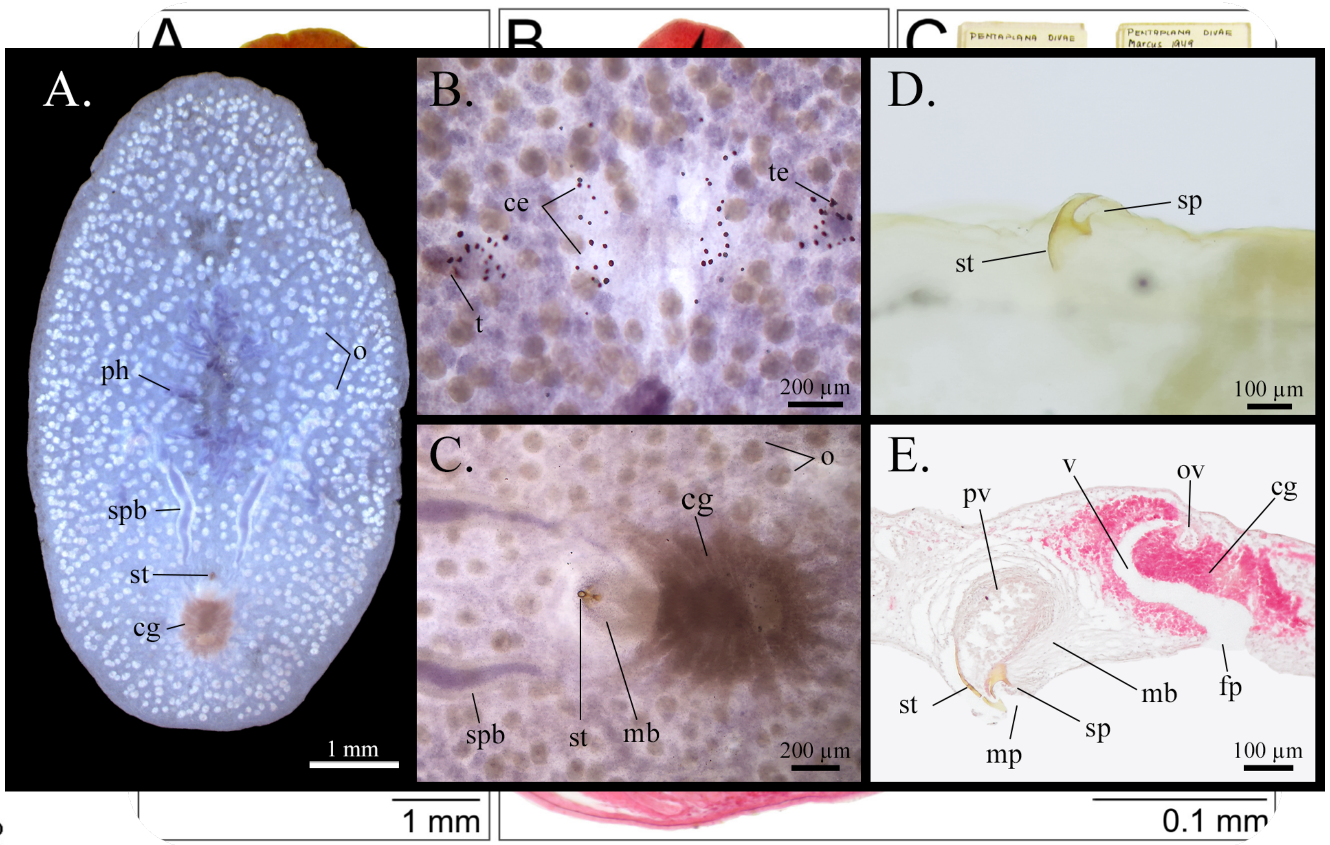
Histology



*Neo-Clear

(Ramos-Sánchez, 2024)

Whole mounts

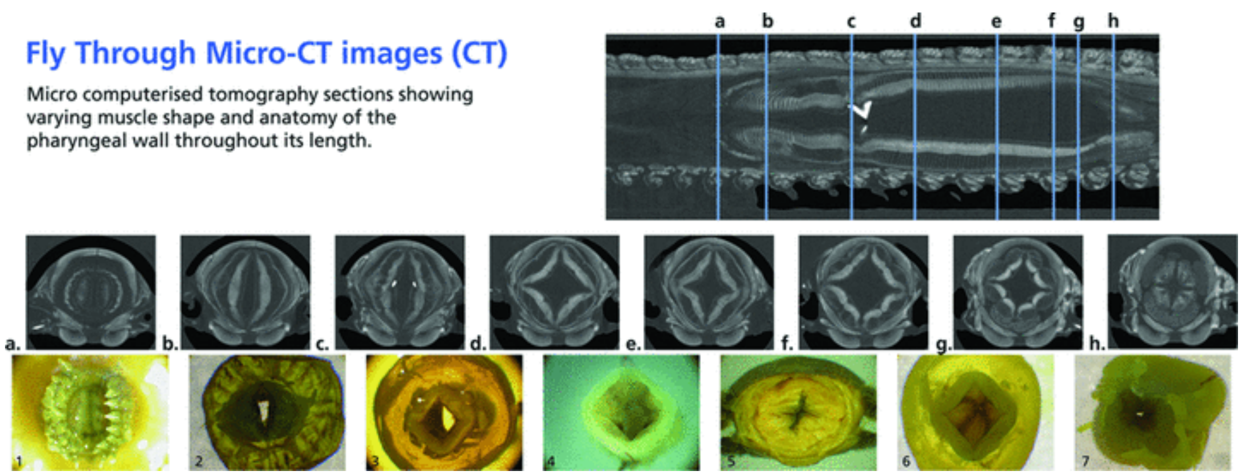


*Methyl salicylate

(Bahia & Schrödl, 2018; Merchán *et al.*, 2025)

Micro-CT scanner as a non-destructive method for identification of polyclads

'Virtual histology'



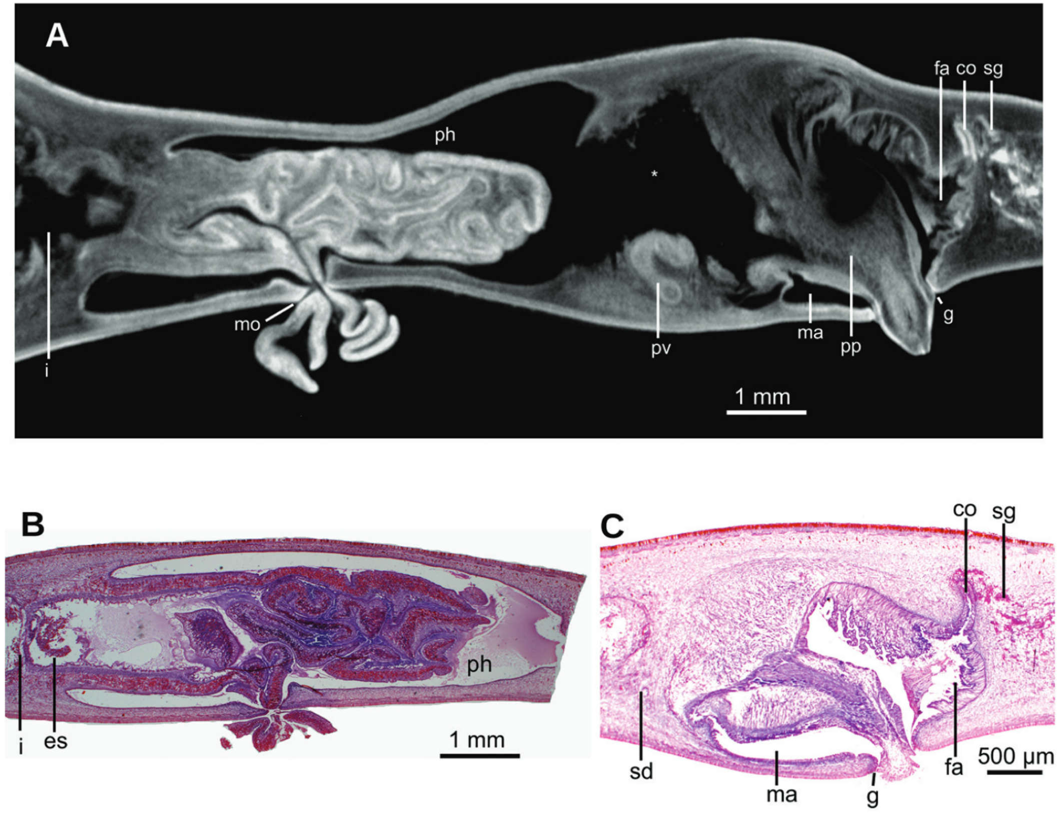


Figure 3. *Obama itatiayana* (Schirch 1929). Syntype C **A** µCT-derived image. Virtual sagittal section of pharynx and copulatory region, pixel size: 6.3 µm **B** photomicrograph of sagittal section of pharynx **C** photomicrograph of sagittal section of copulatory apparatus.

"Traditional techniques for investigating internal morphology require either dissection or the production of serial sections from embedded specimens. (…). However, both techniques are very time consuming and highly destructive…"

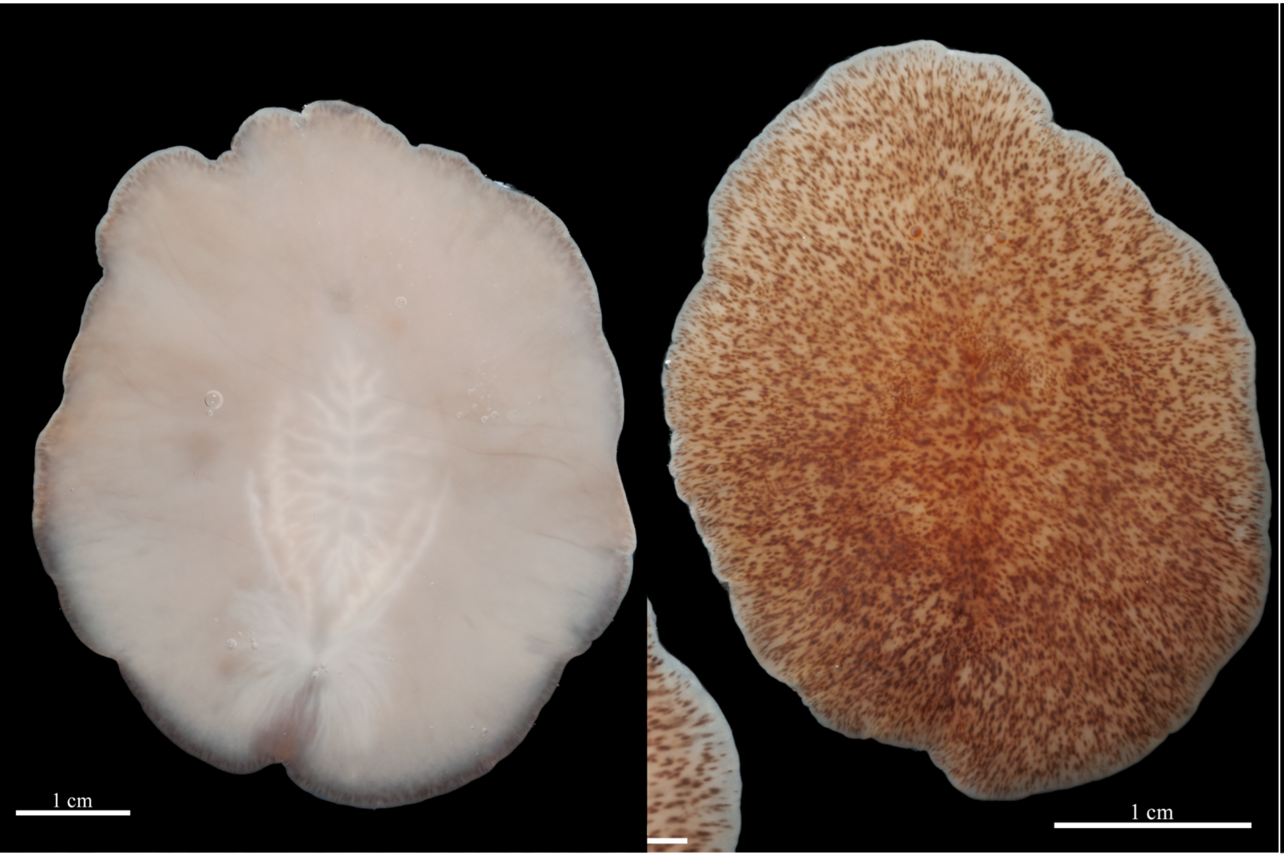
(Dinley *et al*., 2010)

2014)

(Silva & Carbayo, 2020)

Identify polyclad flatworms species through the inspection of the reproductive structures by micro-CT scanning.

Who are you?



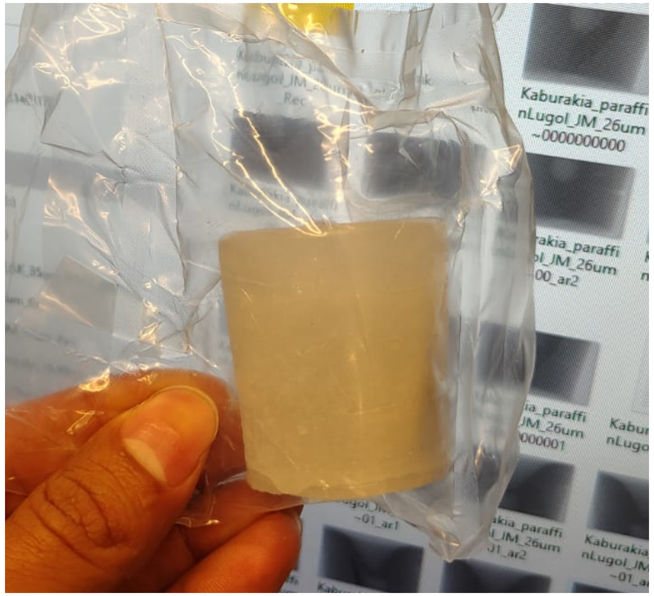
Kaburakia excelsa Bock, 1925

Eurylepta cf. *aurantiaca* Heath & McGregor, 1912

Methodology

Packed alone
Surrounded by jelly
Embedded in paraffin

No Stain
1.25% Buffered Lugol – 2.5% PTA

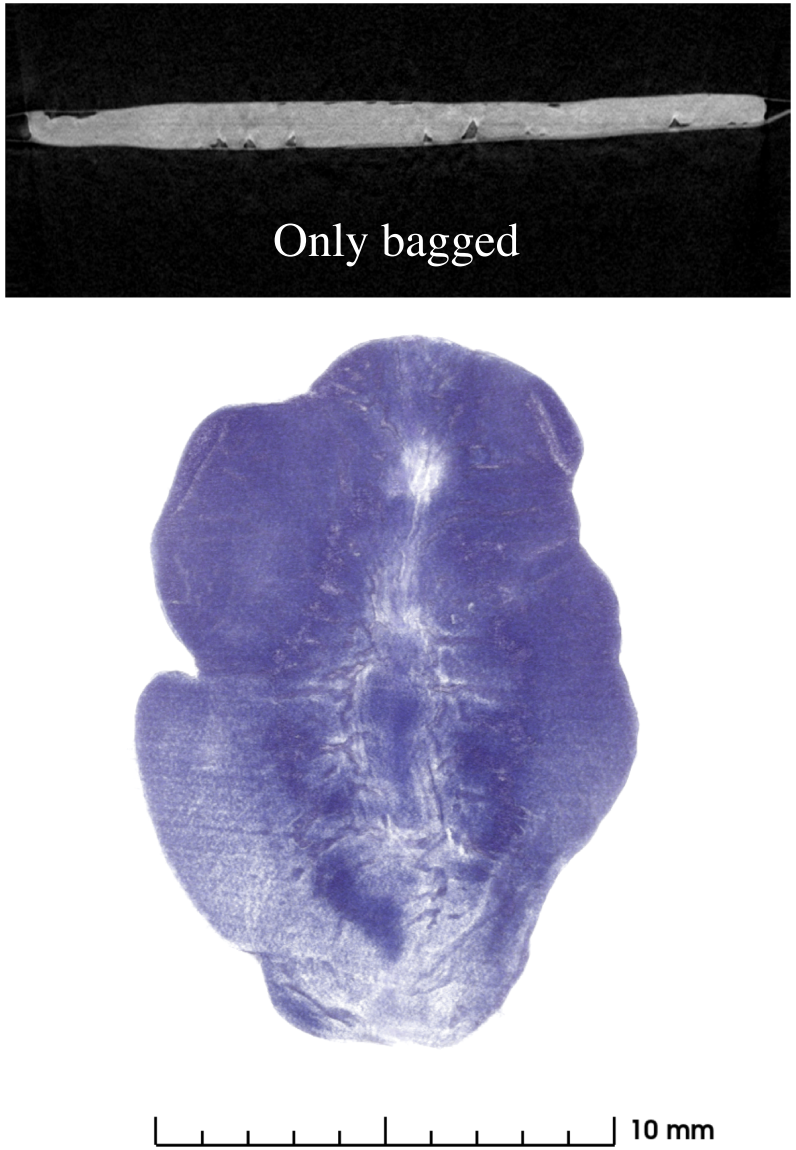


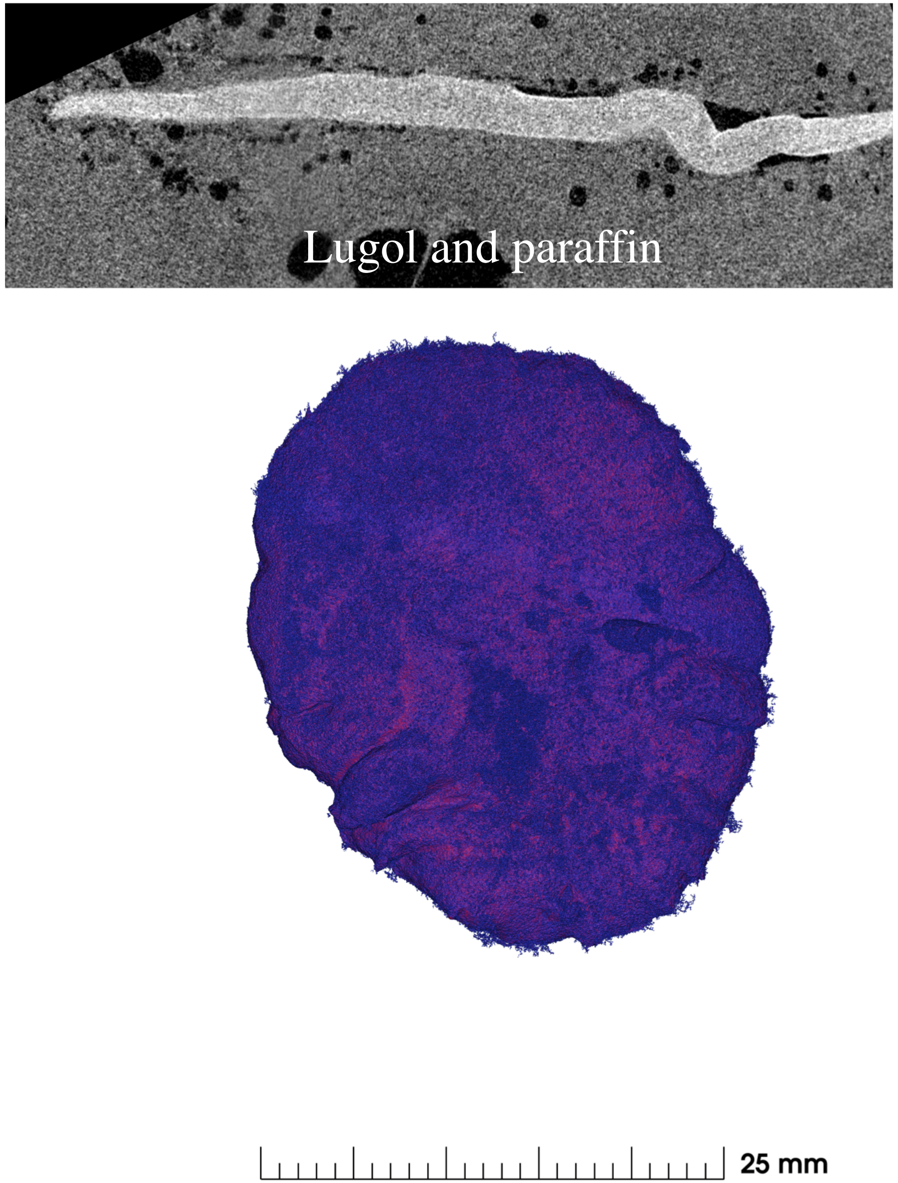
Reconstruction with NRecon → Volume Rendering With 3D Slicer

~7.5% $MgCl_2$ – 10% Formalin

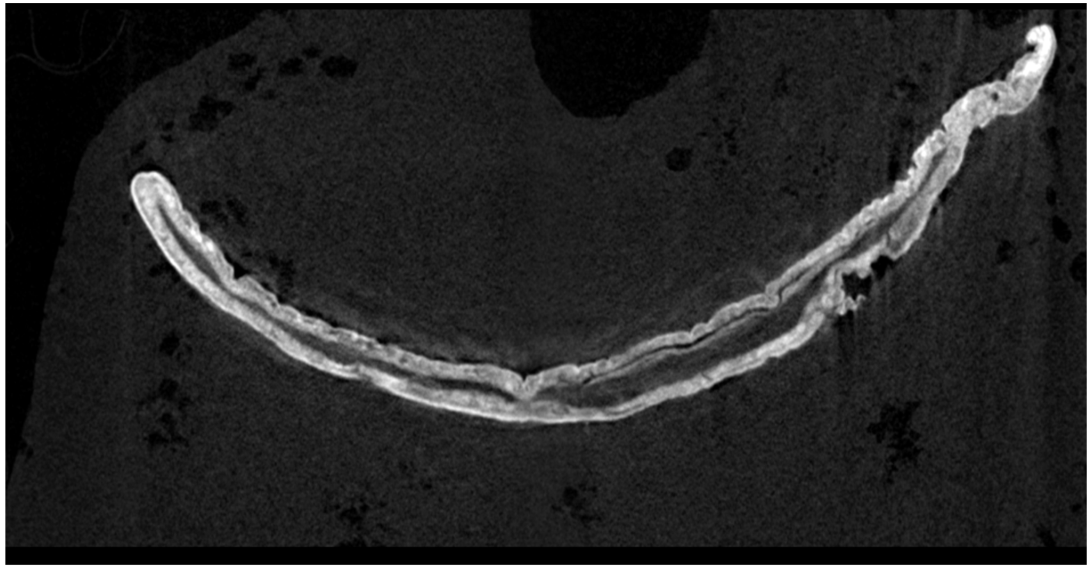
Pixel size between 20-30 μm

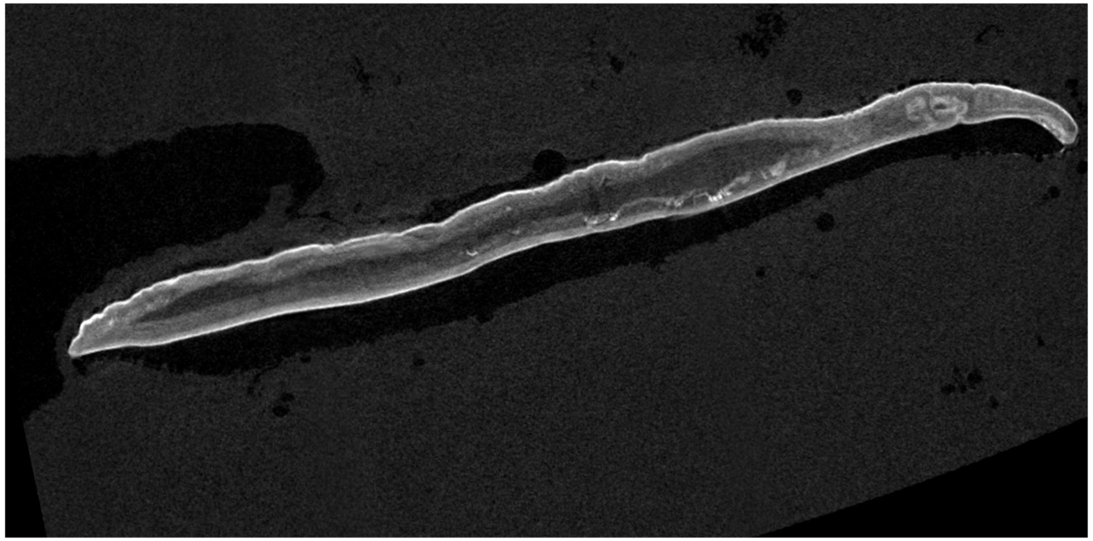
Results



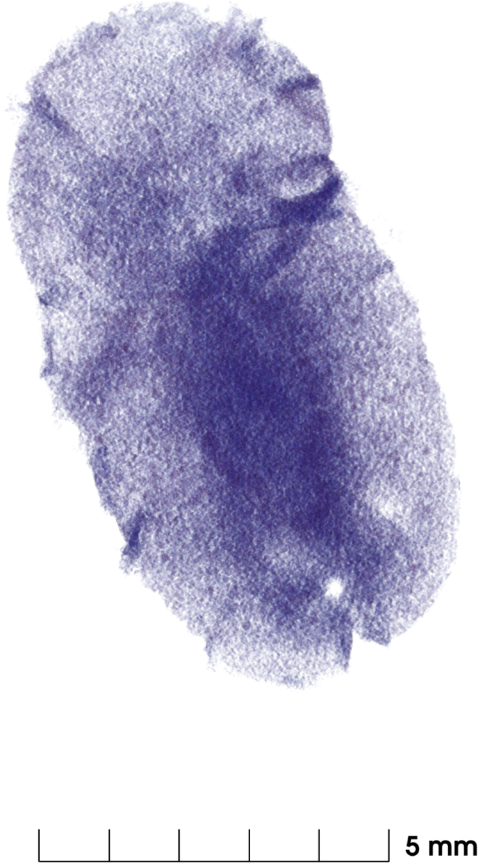


PTA and Paraffin





PTA and Paraffin

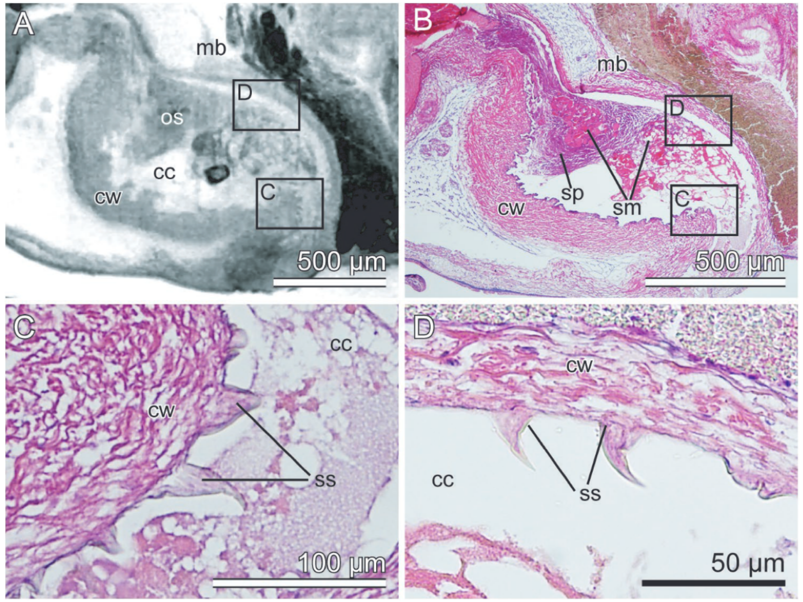


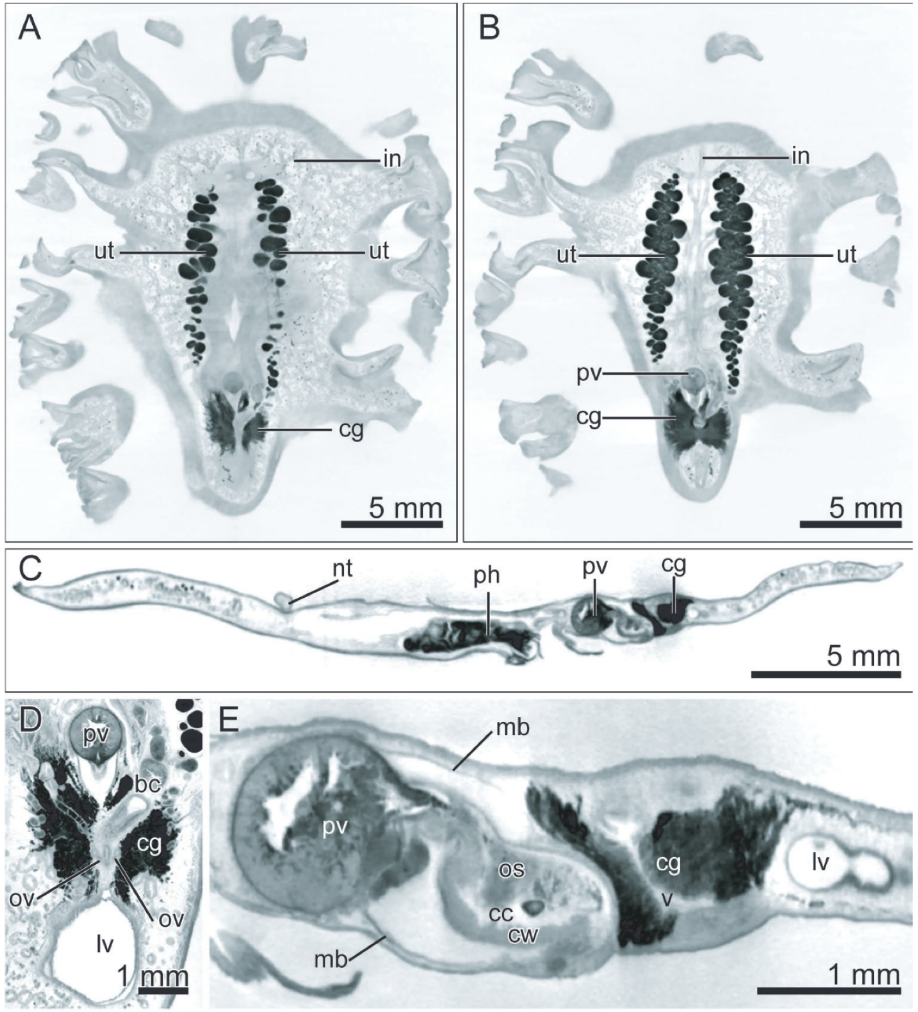
Lugol and Paraffin

It works, but it could be better!

Several variables affect the scan results
and their utility for polyclad identification:

- ➢ The scanner features and limitations
- ➢ The nature/biology of the worms
- ➢ The stain employed
- ➢ ‘Mounting’ the specimens



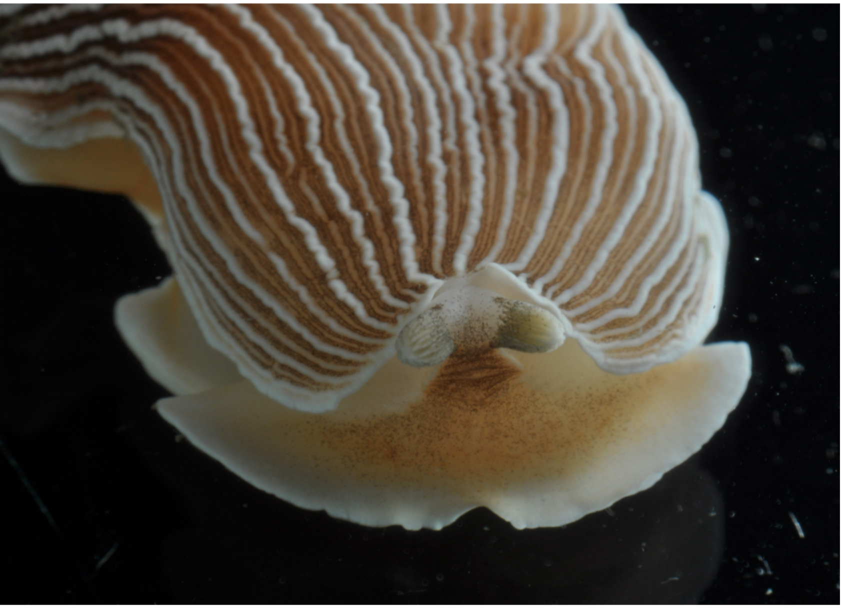


(Oya *et al*., 2024)

Conclusions

- Micro-CT scanning is not yet a standalone tool for polyclad identification, specially for smaller worms
- PTA continues to be the best option for staining soft tissues like those of marine invertebrates for Micro-CT
- Better images may be obtained by further tweaking of the methodologies

MIKU



Thank You

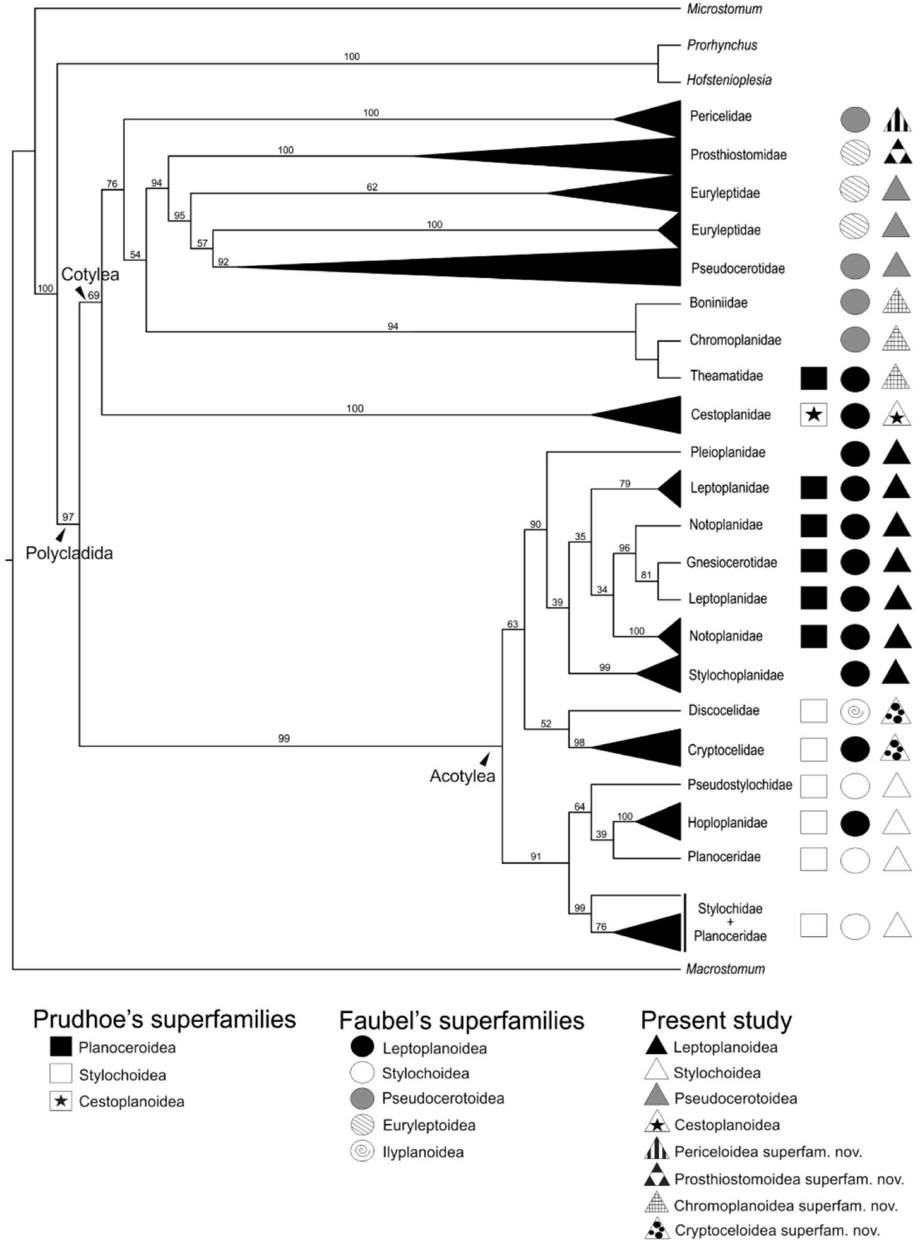
Jorge I. Merchán Mayorga

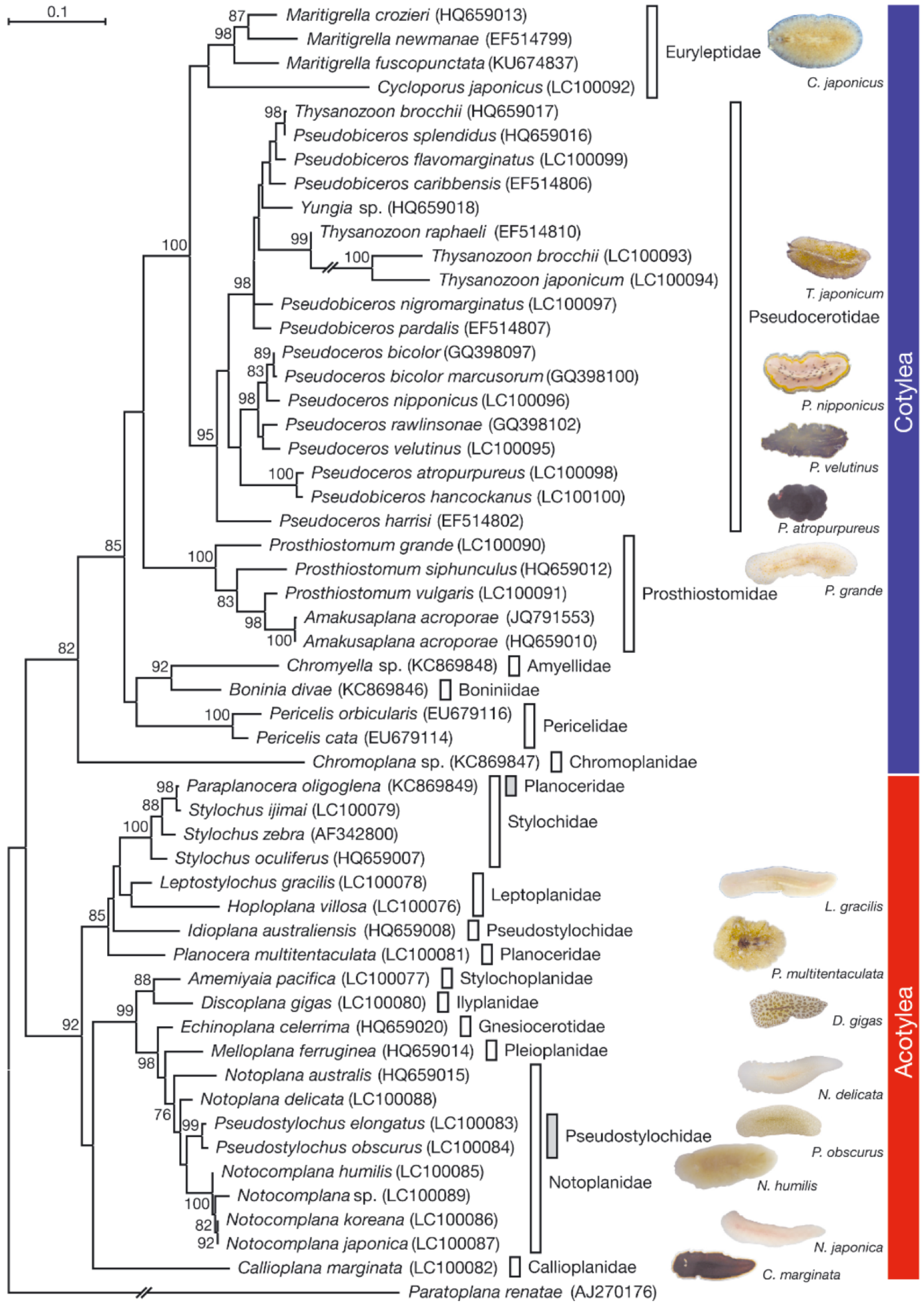
jorgemerchandmc@gmail.com

jorgemerchanim@unimagdalena.edu.co

Referencias

- Appeltans, W., Ahyong, S. T., Anderson, G., Angel, M. V., Artois, T., Bailly, N., Bamber, R., Barber, A., Bartsch, I., Berta, A., Błażewicz-Paszkowycz, M., Bock, P., Boxshall, G., Boyko, C. B., Brandão, S. N., Bray, R. A., Bruce, N. L., Cairns, S. D., Chan, T.-Y., … Costello, M. J. (2012). The Magnitude of Global Marine Species Diversity. *Current Biology*, *22*(23), 2189–2202. https://doi.org/10.1016/j.cub.2012.09.036
- Bahia, J., & Schrödl, M. (2018). Brazilian Polycladida (Rhabditophora: Platyhelminthes): Rediscovery of Marcus' type material and general revision. *Zootaxa*, *4490*(1). https://doi.org/10.11646/zootaxa.4490.1.1
- Bolaños, D. M., Quiroga, S. Y., & Litvaitis, M. K. (2006). A new acotylean flatworm, Armatoplana colombiana n. sp. (Platyhelminthes: Polycladida: Stylochoplanidae) from the Caribbean coast of Colombia, South America. *Zootaxa*, *1162*(1), 53. https://doi.org/10.11646/zootaxa.1162.1.5
- Díaz, J. M., & Acero, A. (2003). Marine biodiversity in Colombia: Achievements, status of knowledge, and challenges. *Gayana (Concepción)*, *67*(2). https://doi.org/10.4067/S0717-65382003000200011
- Dittmann, I., Pérez-Monte, D., Aguado, M. T., Noreña, C., & Egger, B. (2019). Polyclad phylogeny persists to be problematic. *Organisms Diversity & Evolution*, *19*. https://doi.org/10.1007/s13127-019-00415-1
- Faubel, A. (1983). The Polycladida, Turbellaria. Proposal and establishment of a new system. Part I. The Acotylea. *Mitt hamb zool Mus Inst*, *80*, 17–121.
- Faubel, A. (1984). The Polycladida, Turbellaria. Proposal and establishment of a new system. Part II. The Cotylea. *Mitt hamb zool Mus Inst*, *81*, 189–259.
- Litvaitis, M. K., Bolaños, D. M., & Quiroga, S. Y. (2019). Systematic congruence in Polycladida (Platyhelminthes, Rhabditophora): Are DNA and morphology telling the same story? *Zoological Journal of the Linnean Society*, *186*(4), 865–891. https://doi.org/10.1093/zoolinnean/zlz007
- Löbl, I., Klausnitzer, B., Hartmann, M., & Krell, F.-T. (2023). The Silent Extinction of Species and Taxonomists—An Appeal to Science Policymakers and Legislators. *Diversity*, *15*(10), Article 10. https://doi.org/10.3390/d15101053
- Quiroga, S. Y., Bolaños, D. M., & Litvaitis, M. K. (2004). A checklist of polyclad flatworms (Platyhelminthes: Polycladida) from the Caribbean coast of Colombia, South America. *Zootaxa*, *633*(1), Article 1. https://doi.org/10.11646/zootaxa.633.1.1
- Quiroga, S. Y., Bolaños, D., & Litvaitis, M. K. (2006). First Description Of DeepSea Polyclad Flatworms From The North Pacific: Anocellidus N. Gen. Profundus N. Sp. (Anocellidae, N. Fam.) And Oligocladus Voightae N. Sp. (Euryleptidae). *Zootaxa*. https://doi.org/10.5281/zenodo.173981
- Newman, L. J., & Cannon, L. R. (1995). The importance of the fixation of colour, pattern and form in tropical Pseudocerotidae (Platyhelminthes, Polycladida). *Hydrobiologia*, *305*, 141–143.
- Newman, L. J., & Cannon, L. (2003). *Marine Flatworms: The World of Polyclads*. Csiro Publishing. https://doi.org/10.1071/9780643101197
- Oya, Y., & Kajihara, H. (2020). Molecular Phylogenetic Analysis of Acotylea (Platyhelminthes: Polycladida). *Zoological Science*, *37*(3), 1. https://doi.org/10.2108/zs190136
- Prudhoe, S. (1985). *A Monograph on Polyclad Turbellaria*. British Museum (Natural History).
- Rawlinson, K. A. (2008). Biodiversity of coastal polyclad flatworm assemblages in the wider Caribbean. *Marine Biology*, *153*(5), 769–778. https://doi.org/10.1007/s00227-007-0845-3





"Polyclad phylogeny persists to be problematic"

- DNA information is scarce, the firsts 'complete' phylogenies were in 2017
- Barcoding as a tool for taxonomy
- We have done a couple steps forward molecular systematics in polyclads
  - ✓ More sequences
  - ✓ Specific primers
  - ✓ Research on mutation rates for the genes

(Bahia *et al*., 2017; Tsunashima *et al*., 2017; Dittman *et al*., 2023; Goodheart *et al*., 2023)