Institute of Parasitology, Biology Centre CAS
Folia Parasitologica 2022, 69: 026
doi: 10.14411/fp.2022.026

Folia Parasitologica
http://folia.paru.cas.cz

Research Article
OPEN ACCESS

Confocal laser scanning microscopy (CLSM) as a new tool for morphological characterisation of both newly collected and museum voucher specimens of the Trypanorhyncha Diesing, 1863 (Platyhelminthes: Cestoda)

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Abstract: Taxonomic issues within Trypanorhyncha, e.g., the inaccurate light microscopic visualisation of the hook patterns, are solvable by confocal laser scanning microscopy (CLSM). We applied CLSM imaging to study *Trygonicola macropora* (Shipley et Hornell, 1906) and *Dollfusiella michiae* (Southwell, 1929) from *Neotrygon caeruliopunctata* Last, White et Séret from Bali, Indonesia. To illustrate the strength and limitations of CLSM, images of *Otobothrium cysticum* (Mayer, 1842) and *Symbothriorhynchus tigaminacantha* Palm, 2004, both permanent mounts from a collection, were also processed. The CLSM created image stacks of many layers, and edited with IMARIS Software, these layers resulted in three-dimensional images of the armature patterns and internal organs of both species. BABB (benzylalcohol and benzylbenzuolate) clearing was applied to *T. macropora*. We conclude that trypanorhynch cestodes stained with Mayer-Schuberg's acetic carmine permanently mounted in Canada balsam are suitable for CLSM, allowing detailed analyses of museum type-material as well as freshly collected and processed worms. BABB resulted in imaging the testes in detail, suggesting other stains to be used for CLSM in trypanorhynch cestode research. Application of CLSM for studies of other cestode groups is highly recommended.

Keywords: Mayer-Schuberg's acetic carmine, benzyl alcohol/benzyl benzoate (BABB) clearing, taxonomy, fish parasites, *Dollfusiella michiae*, *Otobothrium cysticum*, *Symbothriorhynchus tigaminacantha*, *Trygonicola macropora*, Indonesia, *Neotrygon caeruleopunctata*

A proper taxonomy relies on a precise morphological characterisation (Theisen et al. 2017, 2018, Bray et al. 2019, Theisen 2019) with detailed imaging of the diagnostic features. Molecular analyses are a common tool in current taxonomy and phylogeny, but are not sufficient for the identification and/or description of (new) flatworm species (Palm et al. 2017, Theisen et al. 2017, 2018, Bray et al. 2019, Theisen 2019). Consequently, many manuals and standard procedures for permanent mounting, clearing and staining of the different taxa and respective organs are commonly in use for microscopic examinations (e.g., Klimpel et al. 2019).

The most important morphological features used to identify trypanorhynch cestodes are the characteristic hook patterns of the tentacles as main attachment organs (Palm 2004). Other important external morphological characters are the shape of the scolex with its bothria, the surface ultrastructure with the characteristic microtriches (Chervy 2009) and the presence of the so-called bothrial pits or grooves. Internal organs, e.g., the shape and position of the testes are also important characters. The partial fusion of two or four bothria as well as the surface ultrastructure is difficult to observe in light microscopy (LM), whereas scanning electron microscopy (SEM) can illustrate this fusion, the ultrastructure and the hook patterns, especially in smaller worms (Palm 2008). Due to its high resolution of the surface ultrastructure and high magnification, SEM has been widely used to study trypanorhynch cestodes (e.g., Palm 1995, 1997, 2004, 2008, Morales-Ávila et al. 2019).

Taxonomy in trypanorhynch cestodes often relies on rare type specimens deposited in museum collections, fixed and stained under flattened conditions, and mounted in Canada balsam. Depending on the orientation on the slide, some important diagnostic features are difficult to see and additional material is not available. This prevents the use of scanning electron microscopy or molecular genetics for in-depth analyses of the respective specimens and species. During the 20th century new microscopic instruments

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were invented, such as confocal laser scanning microscopy (CLSM), that results in high-resolution three-dimensional colour images (Mulisch et al. 2015).

Developmental studies and tissue labelling of tapeworms supported by CLSM exist (e.g., Hrčková et al. 1993, Halton et al. 1994, Rozario and Newmark 2015) and taxonomic CLSM studies on flatworm taxa, e.g., Monogenea, are available (Petrov et al. 2015, 2016, 2017, Theisen et al. 2017, 2018, Petrov and Gerasev 2019, Theisen 2019).

Trypanorhynchs deposited in museum collections around the world are commonly stained by Mayer-Schuberg's acetic carmine (e.g., Palm 2004, Palm and Bray 2014). In combination with this stain, CLSM for morphological analyses has so far only been applied to the human trematode *Schistosoma mansoni* Sambon, 1907 (Neves et al. 2003, 2004, 2005, Silva-Leitão et al. 2009).

The cestode fauna of newly investigated blue-spotted stingrays *Neotrygon caeruliopunctata* Last, White et Séret revealed the presence of *Dollfusiella michiae* (Southwell, 1929) and *Trygonicola macropora* (Shipley et Hornell, 1906), while *Otobothrium cysticum* (Mayer, 1842) and *Symbothriorhynchus tigaminacantha* Palm, 2004 were available from museum collections. We applied CLSM for these carmine-stained worms to analyse their armature patterns and segment morphology. The possibility to apply CLSM for taxonomic research for the order Trypanorhyncha is discussed, pointing out its many advantages, e.g., in comparison to other microscopic techniques, resulting in declaring it as suitable and promising for this purpose.

MATERIALS AND METHODS

Seven *Neotrygon caeruliopunctata* were sampled from the fish market Pasar Ikan Tradisional Kedonganan (8.757028S, 115.168389E) on the southern Bali coast in 2017 to examine their cestode parasite fauna following standardised methods (Palm 2004, Palm and Bray 2014). Isolated cestodes were fixed in 70% ethanol for subsequent microscopic analyses.

Trypanorhyncha were stained with Mayer-Schuberg's acetic carmine (Palm 2004, Klimpel et al. 2019) with eugenol as clearing agent. Stained specimens were permanently mounted in Canada balsam. These mounts were examined and pictured under a differential interference contrast (DIC) light microscope (Olympus BX53) equipped with an Olympus UC30 camera and a drawing tube (camera lucida).

During the examination with the DIC light microscope, the samples were identified morphologically and morphometrically. The morphologically important features were measured with the help of an Olympus camera and the CellSens Dimension 1.6 software.

A DMI 6000 CS confocal laser scanning microscope (Arg/Kr: 488 nm, He/Ne: 543 nm) equipped with a TCS SP5 II laser scanning unit (Leica Microsystems GmbH, Wetzlar, Germany) at step sizes of 0.4–1.0 mm between successive scanning planes was used. Therefore, both, Mayer-Schuberg's acetic-carmine-stained specimens mounted in Canada balsam and Mayer-Schuberg's acetic-carmine-stained specimens mounted in glycerol were utilised and compared. For this purpose, the same preparations were used that had previously been examined with the light microscope.

In addition, mature segments were BABB cleared (after Puelles et al. 2016), transferred onto a slide with glycerol for subsequent CLSM examination. An argon laser (Ar, 65 mW with a notch filter of 514, 561 and 633 nm) was used to study the three different types of preparations. The optimum excitation wavelength of carmine was λex = 326 nm and the best emission wavelength was λem = 430 nm. Image stacks of approximately 200 images per figure were created. The image piles were merged and edited with IMARIS 6.40 software (Bitplane, Zurich, Switzerland). The 'Contour Surface' tool, 'Blend' tool and 'Shadow Projection' tool were applied for a closer look to relevant surface structures. Available acetic-carmine-stained and Canada balsam mounted specimens were similarly investigated.

Additional specimens were studied with SEM at the Scanning Electron Centre of Rostock University Clinical Medicine (dehydrated with a graded ethanol series, critical point dried), and coated with gold (sputter coater EM SCD500, Leica) with a sputtering current of 20 mA for 199 seconds in an argon atmosphere. A Merlin VP Compact (Zeiss) microscope was used at a voltage of 5 kV and a working distance of 7.5–8 mm. A resolution of 1000 × 1000 pixels as well as an integration time of 500 µs was preset.

RESULTS

Examination of *Neotrygon caeruliopunctata* from Balinese waters revealed the presence of two trypanorhynch species, *Trygonicola macropora* (Figs. 1, 2) and *Dollfusiella michiae* (Fig. 3). The two taxa were identified based on morphological and morphometric data (Beveridge 1990, Beveridge and Campbell 1998, Palm 2004). Both represent new locality records for Balinese waters and a new host record for *N. caeruliopunctata*. The prepared permanent specimens were deposited in the Berlin Natural History Museum, Germany after examination. The collection numbers are: ZMB E.7659 and ZMB E.7660 for *Dollfusiella michiae* and ZMB E. 7661–ZMB E.7671 for *Trygonicola macropora*.

The identification of the two species was carried out with the use of a light microscope (LM), where the morphological characteristics were also measured. The subsequent examination of the specimens with CLSM confirmed the prior results and indentification. The measurements were nearly identical (length of the metabasal hooks from *D. michiae* measured 8–13 µm with LM and 9–13 µm with CLSM), where the measurement of the hooks was facilitated by the 3D visualisation of CLSM.

A 3D image of *T. macropora* was created (Fig. 1 A). Figures 1B and C show the bothria of *T. macropora* with the tentacles from two different angles of view. A red/cyan shift intensified the three dimensional impression and blue/red 3D anaglyph glasses image the figure completely three dimensional. Figure 1D shows a mature proglottid of *T. macropora* in two different imaging modes. A more transparent image in which the internal organs (testes) can be seen (left), while only the surface of the proglottid can be seen on the right. A proglottid brightened with BABB is shown in Fig. 1E. The internal organs such as testes and ovaries are clearly visible in the figure.

The comparison of the study of hook patterns of *T. macropora* with different techniques is shown in Fig. 2. Figure

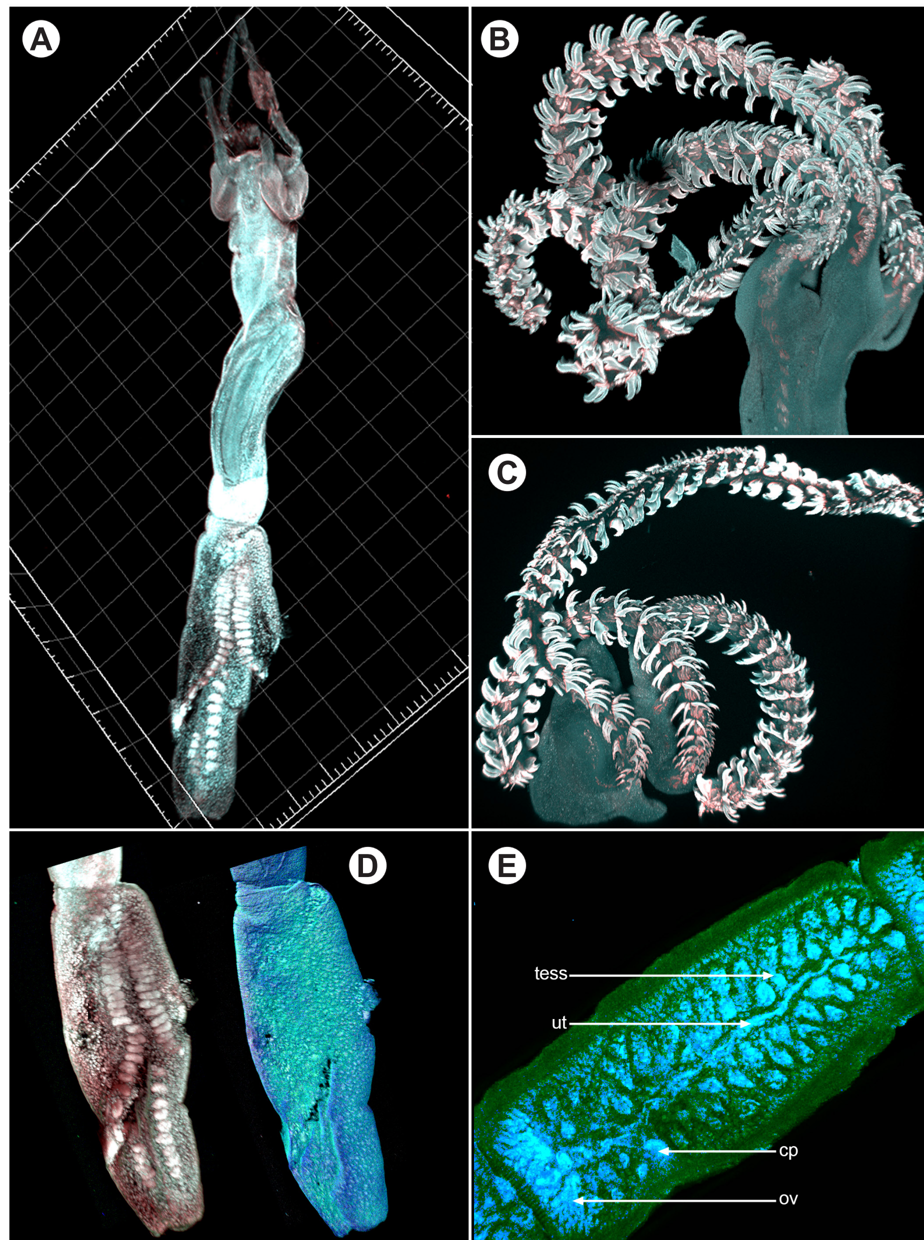

Fig. 1. CLSM pictures of the trypanorhynch cestode *Trygonicola macropora* (Shipley et Hornell, 1906). **A** – Scolex with mature proglottid arranged in a three-dimensional coordinate system; **B** – bothria with tentacles; red/cyan shift, carmine red stained; **C** – bothria with tentacles; red/cyan shift, carmine red stained; **D** – mature proglottid, internal view and surface; carmine red stained; **E** – mature proglottid; BABB cleared. *Abbreviations*: cp – cirrus pouch, ov – ovary, tess – testes, ut – uterus.

2A–C shows the metabasal hooks (captured with CLSM) of *T. macropora* from different viewing angles (tentacles slightly rotated from A to C). A red/cyan shift was applied to enhance the three-D effect with 3D anaglyph glasses in Fig. 2A and B. Figure 2C shows the metabasal hooks without red/cyan shift in a coordinate system. A SEM image of the metabasal hooks can be seen in Fig. 2D. In contrast, Fig. 2E shows a DIC image of the hooks and Fig. 2F visualises the drawing of the hook based on light microscopy.

CLSM images of *D. michiae* are shown in Fig. 3, where Fig. 3A shows the front part of the scolex of *D. michiae* in transparent (Maximum Intensity Projection 'MIP' mode) representation, its tentacle sheaths as well as its surface. The metabasal armature from different angles of *D. michiae* are shown in Fig. 3B–D. The rotation of the tentacles made it easy to measure the hooks. The red/cyan shift enhances the 3D effect with 3D anaglyph glasses, visible in Fig. 3B and C. Figure 4D shows the basal armature without red/cyan shift.

Acetic carmine-stained *Otobothrium cysticum* and *Symbothriorhynchus tigaminacantha* were chosen for comparison. Figure 4 demonstrates the application of CLSM for museum material, which can be studied in new (three-dimensional) dimensions. Figure 4A illustrates *O. cysticum* with a detailed view of the muscular bulbs and bothrial pits. It is noteworthy that especially the bothrial pits, often invisible in LM, are clearly visualised (arrows). Figure 4B shows *S. tigaminacantha*, i.e., views of its scolex surface

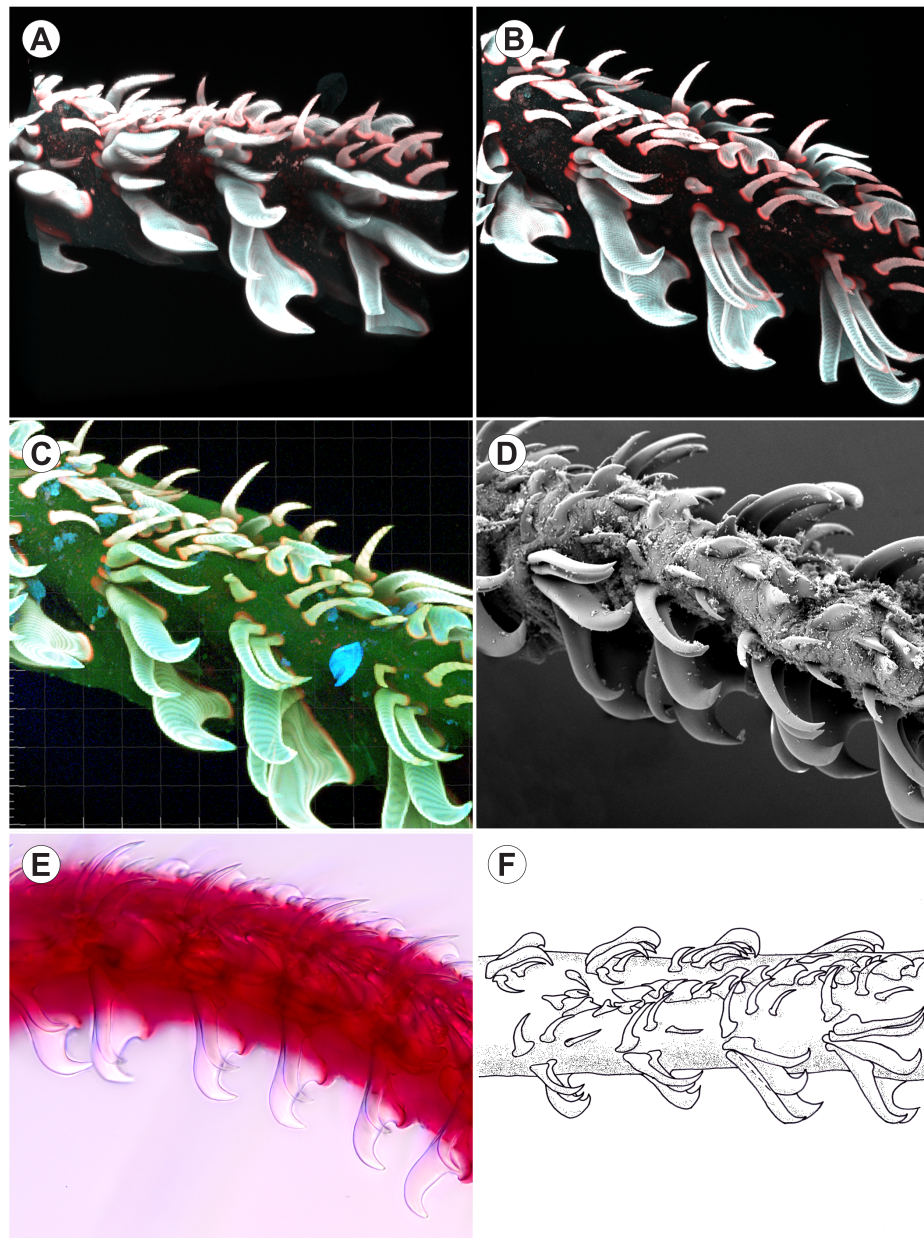

Fig. 2. Various methods to compare the visualisation of the hook patterns of the trypanorhynch cestode *Trygonicola macropora* (Shipley et Hornell, 1906). **A**, **B** – tentacle shown in different angels of view to demonstrate the strength of CLSM to visualise a fixed, mounted worm from various positions, CLSM red/cyan shift, carmine red stained; **C** – CLSM, carmine red stained, arranged in a three-dimensional coordinate system; **D** – SEM; **E** – Light microscopy, carmine red stained; **F** – line drawing.

coloured and arranged in a three-dimensional coordinate system. Figure 4C shows the basal armature of *S. tigaminacantha*. In comparison to Fig. 4C, Fig. 4D shows a light microscopic image of the basal armature of *S. tigaminacantha*. Individual features were measured on a trial basis with CLSM and compared with measurements from the light microscope (LM); no notable differences were observed here (scolex length of *S. tigaminacantha* LM: 1035 µm, CLSM: 1037 µm; bothria length LM: 229 µm, CLSM: 228 µm; bulb length LM: 194 µm, CLSM: 197 µm).

During the preparation and review of CLSM images, no difference was observed between the images that used glycerol or Canada balsam as mounting media. Consequently, this methodological study can illustrate various cestode taxa, complete habitus and specialised structures, the inner organs and the outer surface, applying various filters and colourations, in high magnification and preciseness.

DISCUSSION

In the present study we demonstrate an alternative morphological identification of the trypanorhynch cestodes while applying the confocal laser scanning microscopy (CLSM). For this purpose, results of CLSM, DIC and SEM are herewith briefly compared. In using the ethanol fixed, carmine-stained worms, considered standard for cestode morphology and taxonomy studies, several caveats should be borne in mind. The possible effects of natural variation, variation due to different methods of fixation (particularly

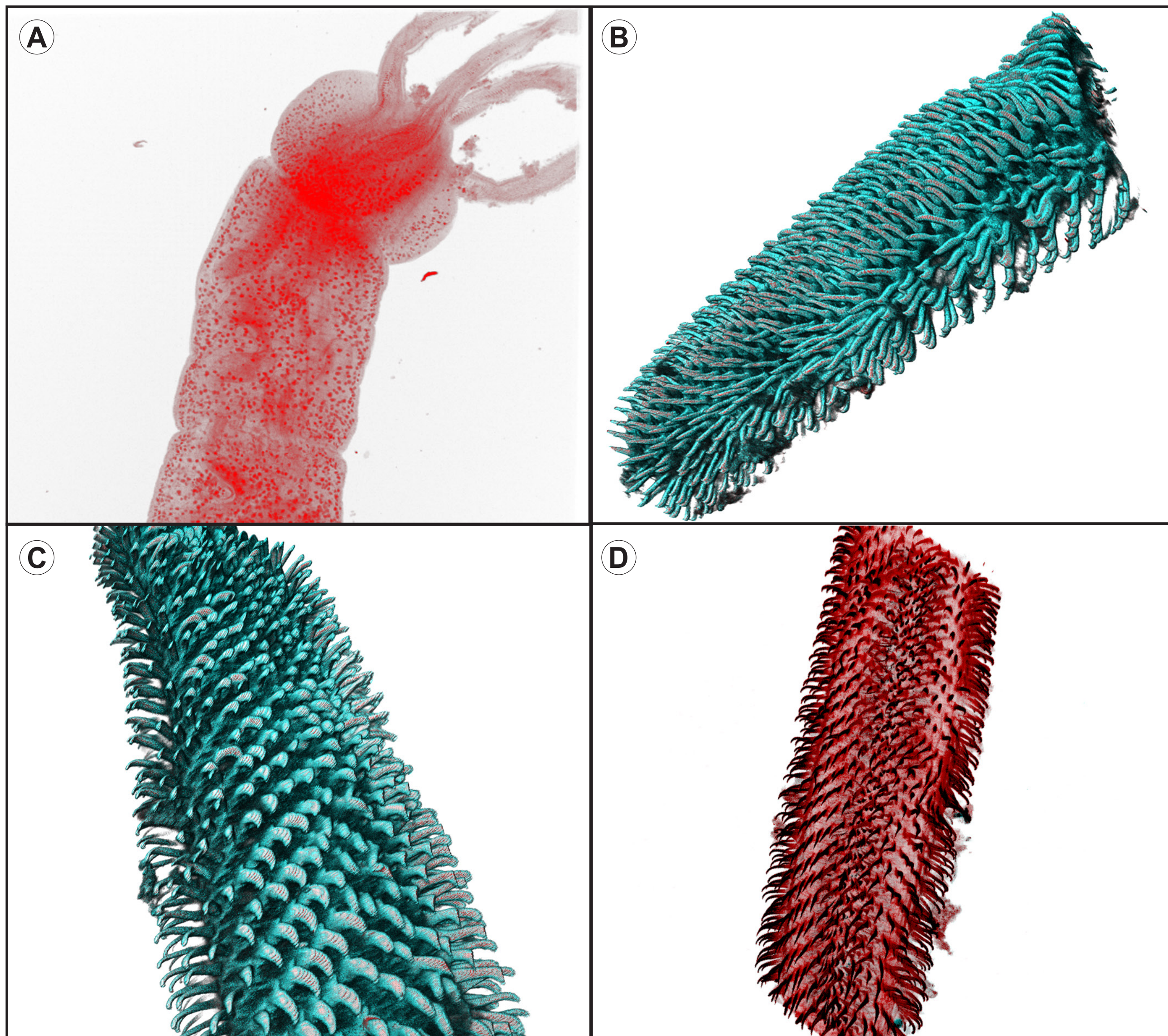

Fig. 3. CLSM pictures from the trypanorhynch cestode *Dollfusiella michiae* (Southwell, 1929), overview and hook patterns. **A** – Scolex overview, carmine red stained, transparent Maximum Intensity Projection (MIP) mode; **B**, **C** – tentacle shown in different angels of view to demonstrate the strength of CLSM to visualise a fixed, mounted worm from various positions, metabasal hooks 'contour surface' red/cyan shift, carmine red stained; **D** – metabasal hooks 'contour surface', carmine red stained.

flattening), different conditions of collection (fresh, frozen, etc.) and allometric growth (Fischthal 1978a,b) should be considered. However, all this has to be considered for other fixatives, stains and imaging techniques as well.

CLSM

The confocal laser-scanning microscope (CLSM) uses fluorescence of specimens (natural or stained) to visualise structures with a laser in one focus plane. It subsequently scans further focus planes and combines them to a three-dimensional object. Consequently, a higher quality of the fluorescence results in a higher image quality. The autofluorescence of most animals is naturally too low for CLSM, but, for instance, some hard structures (e.g., hooks, spicules, eggs) of helminths can be autofluorescent (see, e.g., Forge and MacGuidwin 1989, Degger et al. 2009, Semprucci et al. 2016, Liu et al. 2021). Therefore, specimens must be stained with a fluorescent substance. In trypanorhynch research, Mayer-Schuberg's acetic carmine has been frequently used as the regular staining method for light microscopy (e.g., Palm 2004). Carmine, however, is also used to stain chromatin in combination with CLSM (Stockert et al. 1990). Neves et al. (2003, 2004, 2005) and Silva-Leitão et al. (2009) successfully analysed the Mayer-Schuberg carmine-stained human pathogen *Schistosoma mansoni* Sambon, 1907 (Trematoda) with CLSM. The staining with Mayer-Schuberg solution and embedding in Canada balsam resulted in good fluorescence because the refractive index of Canada balsam (1.52–1.53) resembles that of glycerol (1.45), and both resemble that of the cytoplasm (Ross 1954). During this study, no qualitative differences were found between preparations in Canada balsam or glycerol, so Canada balsam can used as a mounting medium also for CLSM.

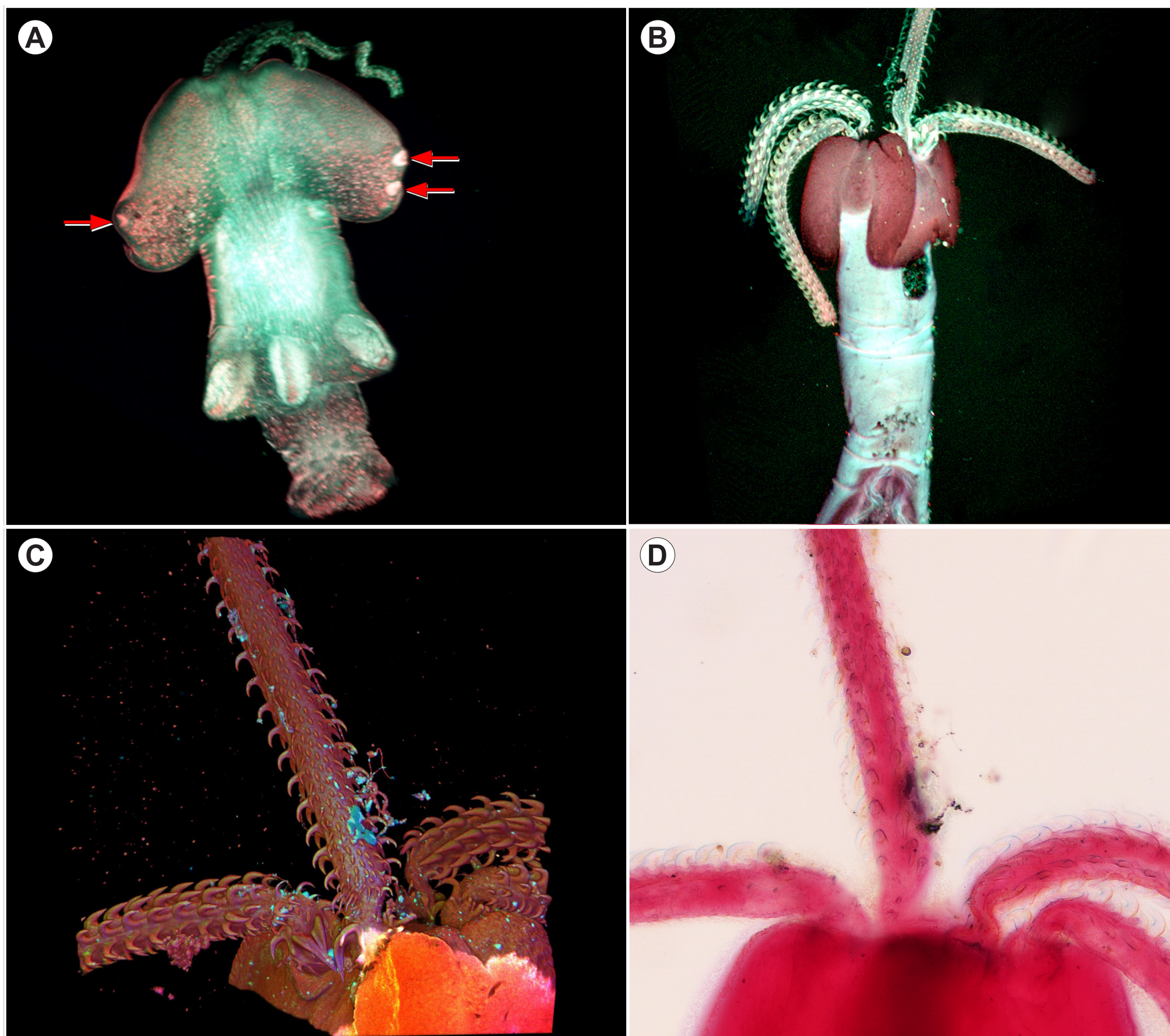

Fig. 4. CLSM and LM pictures of available voucher material of trypanorhynch cestodes, carmine red stained and mounted in Canada balsam. **A** – *Otobothrium cysticum* (Mayer, 1842), scolex with bulbs and bothrial pits (arrows); **B** – *Symbothriorhynchus tigaminacantha* Palm, 2004, scolex with tentacle; **C** – *S. tigaminacantha* basal armature; **D** – light Microscope picture of the basal armature of *S. tigaminacantha.*

Figures 2 and 4 demonstrate the advantages of CLSM compared to DIC light microscopy. Especially the comparison of Fig. 4C and D shows the advantages of CLSM, a clear image of the hook pattern could be created with CLSM. Such a high level of detail and magnification was not possible with the DIC light microscope. Based on the 3D reconstruction, we were able to produce an overview image showing both the external and internal organs. It is also possible to measure hooks based on the 3D reconstruction of the animals by rotating the model in the computer. With the light microscope, imaging of only a single plane is possible, while SEM imaging is restricted to the surface ultrastructure. Often, highlighting the bothrial pits (Fig. 4A) is not feasible with the light microscope. Figures 1B,C, 2A–C and 3 illustrate the tentacles of *T. macropora* and *D. michiae*, respectively. In contrast to light microscopy (Fig. 2E), all hooks per half spiral row were precisely imaged, which makes it easier to recognise the exact armature pattern. However, the magnification and illustration of surface details are not possible compared with SEM. The surface structures in Fig. 1D photographed by CLSM cannot be presented in such detail as the SEM. Photographs by SEM (Fig. 2D) of the surface structures are more accurate and higher magnifications are possible. Similarly, imaging of the internal organs as shown in Fig. 1E and F is not possible in such detail when applying light microscopy. Finally, SEM illustrations are limited to the surface and cannot take pictures of any internal structures.

In addition to acetic carmine-stained specimens, further relevant morphological characters can be examined in particular detail by the use of other staining or clearing substances. BABB (benzylalcohol and benzylbenzuolate) clearing was applied to study the internal organs of the tapeworm proglottids (Fig. 1E). Even though BABB deletes fluorescent proteins, the method is compatible with immunolabeling (Becker et al. 2012, Berke et al. 2016).

BABB has been used, for example, in the study of musculoskeletal tissues (Berke et al. 2016). Figure 1E illustrates a detailed overview of a mature proglottid of *T. macropora*. The testes, ovary, cirrus-sac and other internal organs are clearly visualised. However, these BABB cleared specimens cannot be used for light microscopy because they result in transparent samples.

Although CLSM has many advantages, it will not replace existing microscopic techniques, but rather complement them. For example, preparations (whole mounts) that are too thick cannot be examined with CLSM. Furthermore, the resolution and magnification of the devices is also limited.

DIC light microscopy

Regular light microscopy is fairly limited in the case of delicate and small specimens with complex armature patterns. Under these conditions, DIC light microscopy is a better option because it better differentiates the individual hooks and allows easier interpretation of the armature patterns (Fig. 2E). In both cases, the respective staining of a specimen is of major importance to illustrate the relevant morphological characteristics. For example, trypanorhynchs need intensively stained tentacles for a clearer vision compared to glycerine-cleared material. Well-differentiated proglottids used to identify the internal organs are a result of accurate staining either. This balancing act between staining intensity and differentiation makes it often difficult to analyse all morphological characters based on a single or few specimens. Specimens can be re-stained or cleared, however, thus taking the risk of sample damage. A microscope camera can only focus on a single plane (~ one single layer/level or a small depth of field). Drawings (Fig. 2F) might be also be problematic, their production is time consuming and can be inaccurate or idealised by the 'artist' (Diaz and Valencia 1985).

SEM

A very high magnification and visualisation of, e.g., macromolecules of 1 nm size, is possible with SEM. Consequently, it has been applied to illustrate the surface ultrastructure of trypanorhynch cestodes, including the tentacular armature (Fig. 2D) (Palm 1995, 2004, 2008, Morales-Ávila et al. 2019). One disadvantage is that the specimen used for SEM cannot be analysed for internal structures and cannot be used for any other microscopic technique. In addition, the position of a specimen cannot be altered after fixation onto the adhesive stub (even though the SEM can visualise a fixed sample from various angles of view). Figure 2D also illustrates one of the disadvantages of SEM, which is obtaining clean, mucus-free tentacles of the specimens or otherwise generate figures with characters overlaid by mucus. For these reasons, SEM preparations cannot be used for the description of holotypes or other rare material. Although modern portable SEMs are now available that do not require extensive preparation, they are not commonly used in fish parasitology.

In summary, the three techniques have different advantages and limitations, but in combination are very powerful to describe the morphology of trypanorhynch cestodes, their armature patterns (Fig. 2) as well as external ultrastructure. Compared to the other microscopy techniques, CLSM allows the rotation of the object and thus to examine all sides of the tentacles. The SEM, on the other hand, allows the highest magnification with the highest resolution. The LM produces the weakest images, but by making a drawing, the important features can be worked out. Addition of CLSM to the standard techniques currently in use is recommendable when such a microscope is accessible, and it is possible to apply both, DIC and CLSM microscopy, to one single processed specimen. In addition, acetic carmine-stained and Canada balsam mounted worms can be extracted from the Canada balsam permanent mount, destained and further processed for SEM. It is also of advantage to enhance the three-dimensional impression by a red/cyan shift (application of Imaris software) to easier visualise the bothrial pits of the Trypanorhyncha (Fig. 4A), which may be difficult to detect with the light microscope (Palm 2004). CLSM imaging generally simplifies the identification of such specimens and 3D movies, e.g., with a rotating three-dimensional worm, can be created.

Because CLSM offers a suite of advantages for taxonomists that study the Trypanorhyncha, tapeworms or flatworms, it should be more routinely applied to the taxonomic investigations of flatworms, including tapeworms. It is possible to investigate such platyhelminths with CLSM (see Neves et al. 2003, 2004, 2005 and Silva-Leitão et al. 2009, who showed CLSM figures of carmine red stained *Schistosoma mansoni*). The possibility to create three-dimensional models and movies, e.g., reconstructions of the shape, structures and patters of internal organs, can simplify morphological tasks. This enables the creation of 3D-models of internal organs, e.g., of a mature cestode proglottid, which can add to traditional histological approaches. In times of developing and increasing 3D printing, this might further lead to the possibility to print educational, highly magnified artificial sculptures of worms with their inner organs in future. Also, further 3D remodelling is possible but needs better software access and practice in applying all software settings.

Further possibilities to use CLSM in other platyhelminth taxa, in combination with different staining techniques, should be tested. BABB clearing allows a closer look at the internal organs, whereas other stains might enable a closer look at surface structures like microtriches or bothria. Furthermore, the autofluorescence of the Trypanoryncha should be investigated as there are no studies on this. So far, only a few trypanorhynchs have been studied but the results, although limited, are very promising, especially for the possible use of old acetic carmine-stained specimens. We suggest the direct sorting of collected material for molecular (fixation in 99.6% ethanol) and morphologic (hot water relaxation and/or 70% ethanol fixation; see Cribb and Bray 2010) purposes. The material for morphological analyses should be in good conditions, with damaged animals, body parts or the blastocyst to be used for molecular analyses. Some worms should be stained with Mayer-Schuberg's acetic-carmine and mounted in Canada

balsam in order to add the possibility of CLSM investigations in future.

Acknowledgements. This is publication no. 14 under the Memorandum of Understanding between the Faculty of Veterinary Medicine, Udayana University, Bali, and the Faculty of Agricultural and Environmental Sciences, Aquaculture and Sea-Ranching, University Rostock, Germany, promote fish parasite and biodiversity research in Indonesia. We are thankful to Stefan Richter, Christian Wirkner and Thomas Frase from the Institute of Biosciences, General and Systematic Zoology, University of Rostock, for providing the opportunity and assistance to work with CLSM. The confocal microscope was jointly sponsored by the Deutsche Forschungsgemeinschaft and the Land Mecklenburg-Vorpommern (DFG INST 264/70-1 FUGG). We also thank Marcus Frank, Armin Springer and Karoline Schulz from the Electron Microscopy Centre, University of Rostock, for assistance to work with the SEM.

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Received 20 October 2021 Accepted 22 July 2022 Published online 10 November 2022

Cite this article as: Neitemeier-Duventester X., Bick A., Theisen S., Palm H.W. 2022: Confocal laser scanning microscopy (CLSM) as a new tool for morphological characterisation of both newly collected and museum voucher specimens of the Trypanorhyncha Diesing, 1863 (Platyhelminthes: Cestoda). Folia Parasitol. 69: 026.