period (Heil 2009). Alternatively, 10% formalin can be used to fix samples for 24-48 hours, followed by replacement of the fixative by 70%, 80%, 90% and 96% ethanol (each concentration for one hour). Samples can be kept in 96% ethanol in the freezer for a longer period. Afterwards, the samples are embedded in paraffin and cut into slides that can later be stained by haematoxylin-eosin or Giemsa. When fixing the samples, do not forget to label each vial with a tissue sample on the outside and by inserting a label with the code of the host written in pencil.

12. Fix another small part of the infected organ in 96-99% ethanol or, for longer sample storage, in TNES urea buffers (Asahida *et al.* 1996) for subsequent DNA extraction and molecular characterisation of the parasite.

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3.3.3. ECTOPARASITIC HELMINTHS (MONOGENEA)

Eva ŘEHULKOVÁ

Introduction

Monogeneans are common, almost exclusively, ectoparasitic flatworms of freshwater, brackish water and marine fishes. Most monogeneans are tiny, which makes their sampling and further processing more difficult compared with larger-sized endohelminths such as most tapeworms, acanthocephalans or nematodes. Species identification of monogeneans may be difficult and its accuracy depends, to a large extent, on the quality of the material available. Therefore, adequate methods of sampling and processing monogeneans are required. If monogeneans are not collected and fixed correctly, it may affect the reliability of the morphometric data on taxonomically important structures.

Examination of fish for monogeneans

Fish should be examined immediately following their death while the monogeneans are still alive because living monogeneans are more easily detected by their movements. In addition, observations of living parasites may yield valuable information on internal structures (*e.g.*, digestive and excretory system) and the natural configuration of sclerotised hard parts. *Post-mortem* changes of monogeneans, which usually disintegrate quickly after they die, might make taxonomical evaluation of the specimens collected difficult or even impossible. The only disadvantage of collecting the living monogeneans is that they are sometimes harder to isolate because they are difficult to mount and orientate on a slide.

Fixed or preserved fish should be studied in a similar way as described below, but the quality of the specimens obtained is always much worse compared with fresh material; in some cases, a reliable identification of the worms cannot be made. It is important to point out that the surface of the fish should be kept wet during any manipulation and handling of the fish (taking photos, measurements, tissue samples, etc.), because drying up results in the damage or loss of monogeneans on the skin and fins. Therefore, the surface organs (skin, fins, nostrils, mouth and gill cavity) must be examined first after all the necessary data are recorded (see Chapter 3.3.1).

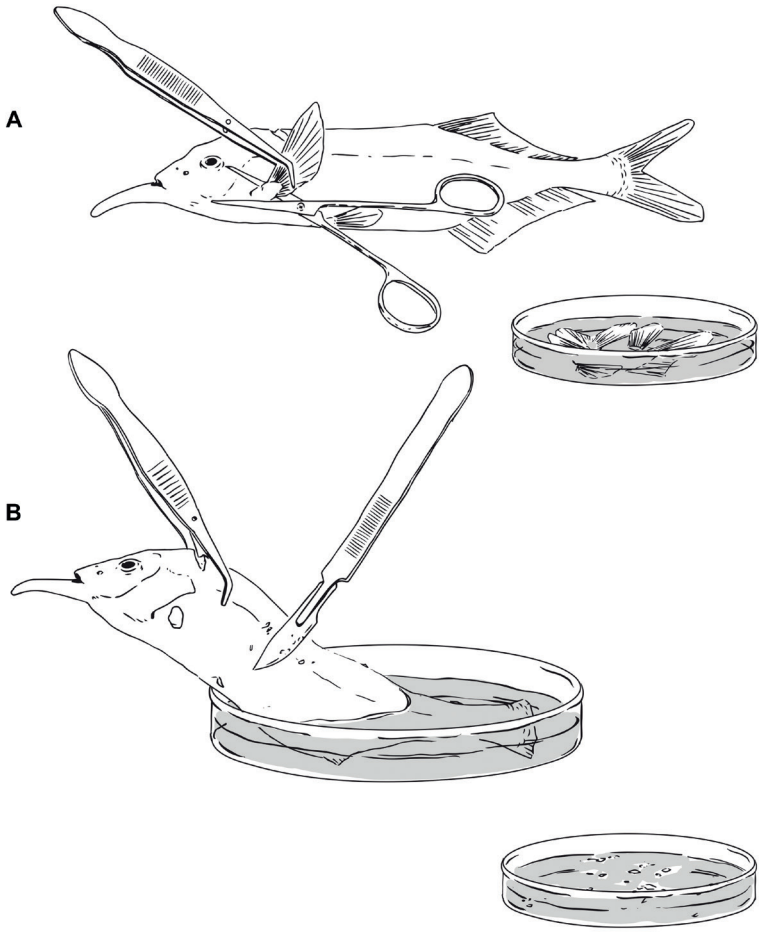

Fig. 3.3.3.1. Examination of fish for monogeneans. **A.** Cutting off fins; **B.** Scraping off mucus. (Illustration by M. Luo and E. Řehulková.)

PROCEDURE

1. Kill the fish using approved methods of euthanasia if it is not dead (*e.g.*, bought at the market or dead after capture).

2. Holding the fish with forceps, cut off the fins using scissors and place them in a Petri dish with water (preferably site water, *i.e.*, from the same source as the fish) (Fig. 3.3.3.1A).

3. Using a scalpel or slide, gently scrape mucus from the whole surface of the fish into a Petri dish with site water (Fig. 3.3.3.1B). If the fish is small (less than 10 cm), examine the whole fish directly under a dissecting microscope (magnification 20×). In this case, an upper illuminator for incident light viewing is required.

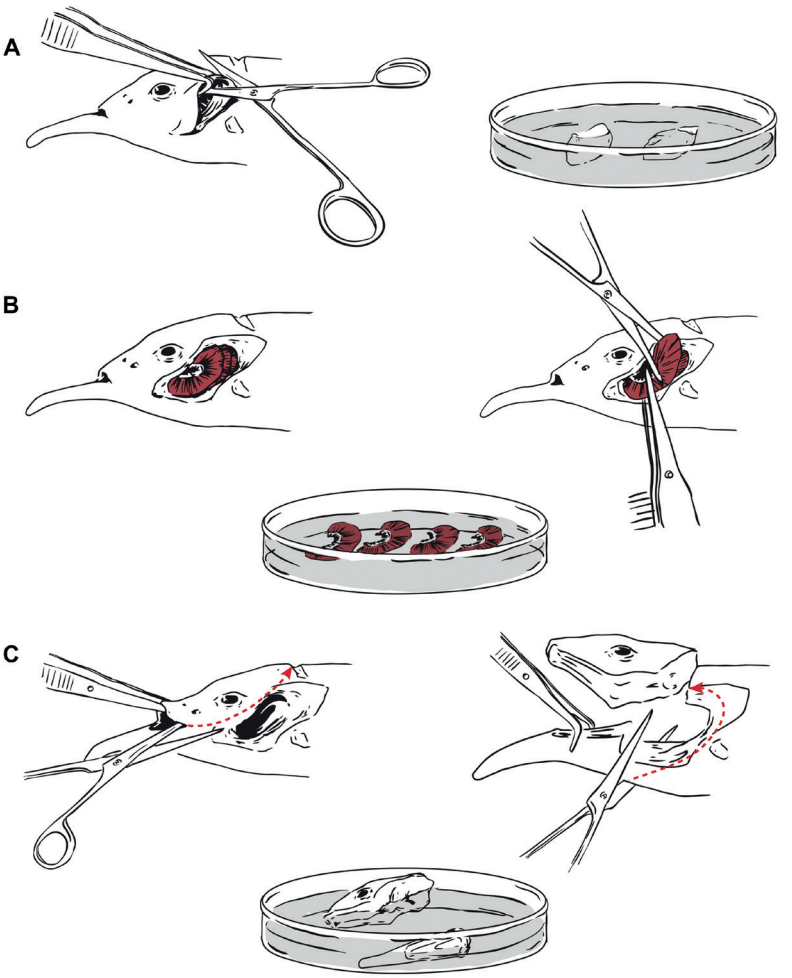

Fig. 3.3.3.2. Examination of fish for monogeneans. **A.** Removing of operculum; **B.** Extraction of gill arches; **C.** Separation of the upper part of the head from the lower part. (Illustration by M. Luo and E. Řehulková.)

4. Remove the operculum of the fish with scissors (Fig. 3.3.3.2A), cut off the gill arches (one by one) from the gill cavity and transfer them to a separate Petri dish with site water (Fig. 3.3.3.2B). If microhabitat preference is studied, each Petri dish should be labelled with the side/number of the gill arch (ideally 1 to 4 from external to internal).

5. Separate the upper part (nostrils, mouth) of the head from the lower part (mouth, pharynx, gill cavity); cut the mouth on both sides of the head towards the oesophagus (scissors following the dorsal side of the pharynx), decapitate the fish just behind the opercula, and place both parts directly in a separate dish with site water (Fig. 3.3.3.2C).

6. Carefully examine the mucus and all organs in Petri dishes with the aid of fine needles under a dissecting microscope at about 20× magnification. Check also the water in each Petri dish for detached monogeneans.

7. Carefully remove each worm from host tissues and place it in a drop of water on a slide, where it is can be fixed immediately (see below) or observed *in vivo* and photographed if the microscope is equipped with a digital camera.

8. After monogeneans from surface organs including gills are collected and fixed, the internal organs should also be examined for endoparasitic monogeneans (*e.g.*, species of *Enterogyrus* in the stomach of cichlids).

Fixation of monogeneans

A variety of methods are used to preserve monogeneans on slides, but some of them do not provide permanent preparations suitable for a deposition in museum collections as types (if a new species is described) or vouchers (faunal surveys and ecological studies). Basically, there are two methodological approaches to processing these parasites. The first one is focused on a study of sclerotised structures, the second one on observations of soft internal structures. To obtain the best results from both these approaches, two different preparation techniques should be used.

For a study of sclerotised structures the method of 'completely flattening' specimens is applied, where monogeneans are flattened under coverslip pressure until their body wall ruptures (see Fig. 3.3.3.3). Using this method, the vitelline follicles disintegrate after the rupture of the body and do not hamper observation of the male copulatory organ and vagina. If monogenean specimens are not sufficiently flattened, the shape of sclerotised structures may not be properly interpreted and their measurements tend to be shorter because of their twisted position. In contrast, coverslip pressure may affect the actual orientation of sclerotised structures with respect to the body axis. For that reason, the orientation of taxonomically important structures should be taken from non-flattened stained specimens.

Fixation to study sclerotised structures

To study the sclerotised structures of the haptor and the distal parts of the reproductive system (*i.e.*, male copulatory organ and vagina), the methods (formalin-glycerine fixative), proposed by R. Ergens in 1956 (in a Czech-written unpublished technical report) and later corroborated by Malmberg (1957; glycerine-ammonium picrate fixative or GAP), should be used. Formalin-glycerine fixative is prepared by mixing five parts of 4% formaldehyde solution and one part of glycerine/glycerol. GAP is prepared by mixing one part of saturated ammonium picrate solution and one part of glycerine.

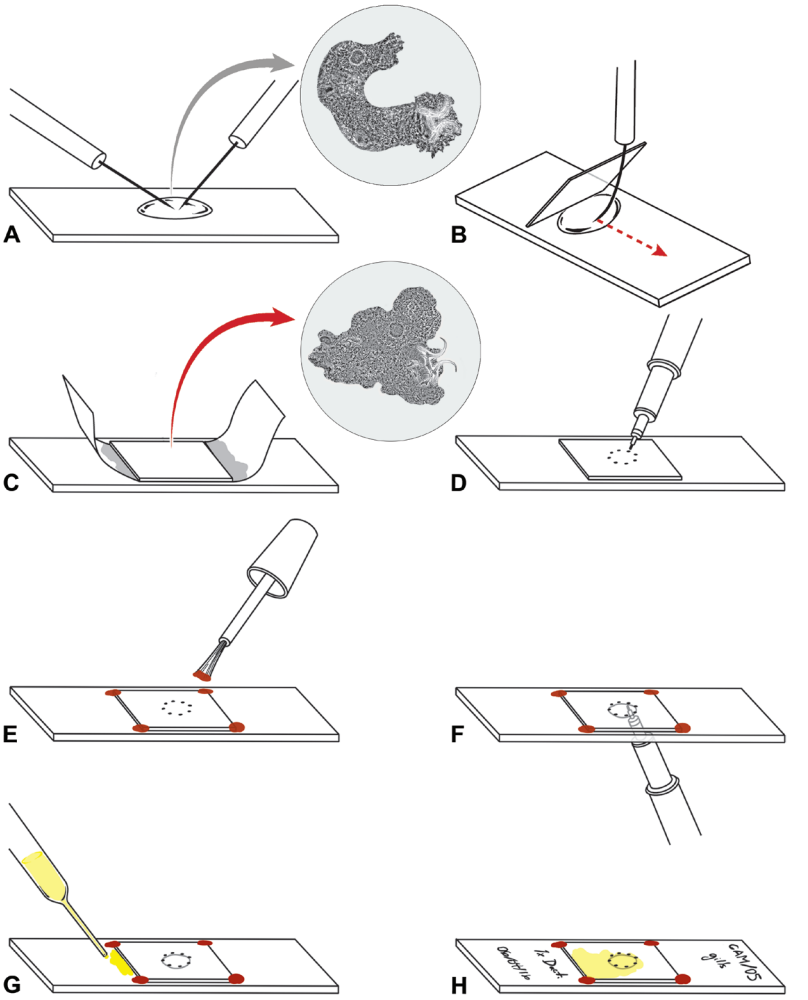

Fig. 3.3.3.3. Slide preparation of monogeneans mounted in GAP (glycerine-ammonium picrate) for subsequent morphological examination of the sclerotised structures. (Illustration by M. Luo and E. Řehulková.)

PROCEDURE (Fig. 3.3.3.3)

1. Place at maximum five clean worms, *i.e.*, worms without host tissue, mucus or any debris, which should be removed using fine needles, in a water drop on a slide using fine needles.
2. Lay a coverslip on the worm(s) while observing its/their position under a dissecting microscope to avoid the loss of the worm(s).
3. Remove excess water from under the coverslip by placing a piece of filter paper at the edge of the coverslip (best from both sides), thus further flattening the specimen(s) until the body wall ruptures.

4. Under the dissecting microscope, gently mark the position of the worm(s) by a dotted circle on the upper side of the coverslip.
5. Seal all four corners of the coverslip with Noyer's lacquer or nail varnish.
6. Trace the dotted circle (using an ethanol-resistant pen) around the worm on the reverse (lower) side of the slide.
7. Add a small drop of formalin-glycerol fixative (or GAP) on the edge of one side of the coverslip. Avoid adding a large volume of fixative as it can lift the coverslip and the flattened/ruptured worm(s) will disintegrate.
8. Label the slide (using an ethanol-resistant pen) with a field number (unique code) of the fish examined, date of collection, infection site (on the host), higher-rank taxon name (usually family) to which the specimen belongs (if known), or unique code of the worm (if part of it was fixed separately for subsequent DNA analysis).
9. Leave the slide on the table in a horizontal position to saturate the worm with formalin-glycerol (or GAP) for a couple of hours (overnight) before storage.
10. Seal the coverslip with enamel paint (nail varnish or Canada balsam) to prevent the mount from drying out.

Since both formalin-glycerine fixative and GAP are semi-permanent mediums, it is necessary for long-term storage, including deposition in museum collections, to remount these preparations using the method of Ergens (1969). This method produces permanent mounts, but some worms may be lost during the remounting procedure, especially if they are broken when the coverslip is detached. It is therefore strongly recommended to make drawings and take measurements from formalin-glycerine or GAP-fixed specimens before remounting them; another option is to take a photo as a photo-voucher.

Fixation to study the soft structures and further processing

To observe the soft internal structures, monogeneans should be relaxed during fixation and then stained with appropriate stains. Fixation with a fixative at ambient temperature (4% formalin or 70% ethanol) is useful only when monogeneans are being (moderately) flattened under a light coverslip pressure. This is best accomplished by placing the worms in a drop of water in a small Petri dish and covering them with a coverslip with a small weight on top (*e.g.*, a metal bolt or nut of approximately 2 g). It is important to note that too much pressure will distort the arrangement/size of the internal organs. Fixation with a hot fixative can avoid this disadvantage. Using hot 4% formalin is the best option (similarly as for trematodes, tapeworms and nematodes – see Chapter 3.3.4), because it penetrates fast into tissues and makes them well-preserved and more suitable for staining compared with samples fixed using hot water. If heating formalin is a practical problem, hot water can be used as described by Justine *et al.* (2012). Hot-water fixation makes it possible to use the worms for both morphological observation (after fixing with 4% formalin or 70% ethanol and subsequent staining) and DNA sequencing (fixed worms are immediately placed in molecular grade 96-99% ethanol).

Fixation of monogeneans for molecular studies

Even though the identification of monogeneans is based mainly on morphological characteristics, molecular data are important for taxonomic, phylogenetic and ecological studies. Therefore, it is strongly recommended always to fix some (parts of) specimens (see below) for genetic analyses (DNA sequencing). Simultaneous infections of fish with several, morphologically similar species represent a serious obstacle in molecular studies because the identity of sequenced worms cannot be ascertained without the availability of a corresponding morphological voucher, *i.e.*, hologenophore (see Pleijel *et al.* 2008 for terminology). In this case, the worms should be divided into three parts; the anterior body part comprising the male copulatory organ and the posterior part with the haptor are prepared for morphological observation as described above (*i.e.*, fixed with formalin-glycerine or GAP), whereas the middle part of the body is fixed in molecular-grade ethanol. However, this procedure is often inapplicable due to the small size of most monogeneans (*i.e.*, species of the families Dactylogyridae and Gyrodactylidae). Therefore, worms are cut just into two parts; that part which enables species-level identification (the posterior part with the haptor in gyrodactylids and diplozoids, the anterior part with the male copulatory organ in dactylogyrids) is fixed for morphological study and the remaining half of the body is fixed for molecular work.

It is important to note that only live or ethanol-fixed monogeneans are suitable for molecular studies. Formalin-fixed worms should not be used because their DNA is fragmented or considerably damaged. The procedure for dividing worms for both morphological and molecular studies is briefly described below.

PROCEDURE (Fig. 3.3.3.4)

1. Place the living or ethanol-preserved worm in a drop of water on a slide.
2. Under a dissecting microscope, divide the body of the worm into two parts using fine needles.
3. Transfer half of the body which does not contain the most important diagnostic structures, to an Eppendorf tube with molecular grades, *i.e.*, non-denaturated 96-99% ethanol and, if possible, store the sample in a refrigerator or freezer.
4. Fix the rest of the body in formalin-glycerine or GAP under coverslip pressure (if the worm is alive) or with Hoyer's medium, as described below.
5. Use identical labelling for the tube and slide to match the morphological voucher (hologenophore and paragenophores) with the sample to be sequenced.
6. After morphological evaluation, deposit the hologenophore in an internationally accessible collection, ideally together with type (holotype, paratypes) or voucher specimens from the same host.

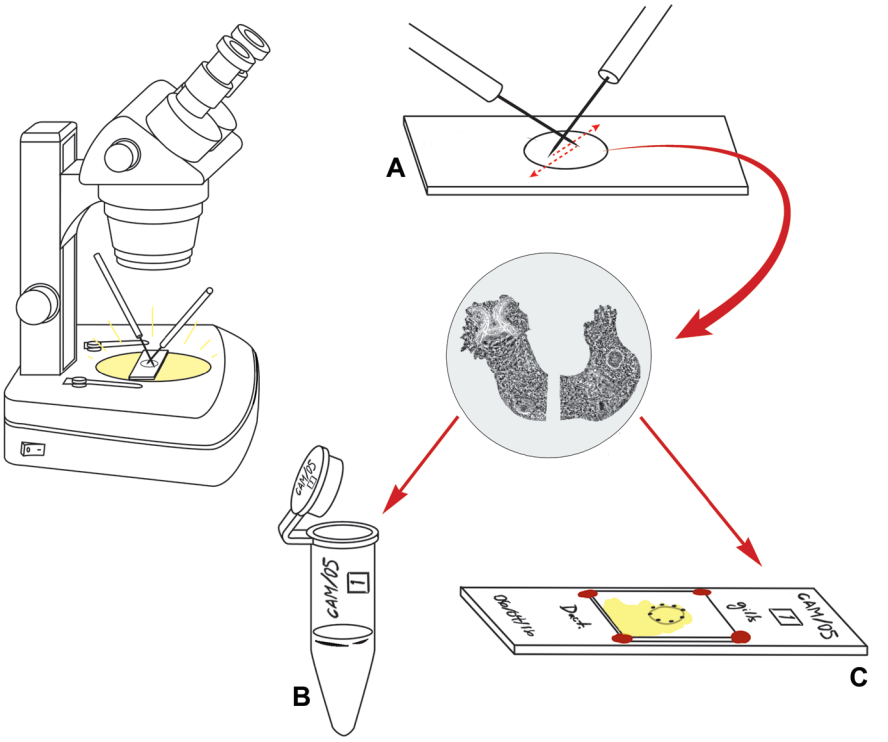

Fig. 3.3.3.4. Collection and identification of specimens for DNA analyses: specimen bisection using fine needles. (Illustration by M. Luo and E. Řehulková.)

Processing of fixed/preserved monogeneans

Study of sclerotised structures

Fixed/preserved monogeneans in vials are observed after being placed into a drop of water on a slide, removing excessive water and mounting them in Hoyer's medium. The slides should be kept in a horizontal position until the medium had solidified. As the worms are cleared rapidly, sclerotised structures and internal organs can be readily observed. Since this is a semi-permanent medium, it is best to ring the coverslip with enamel paint (or Canada balsam) after the medium has solidified. Hoyer's medium is prepared by mixing 30 g Arabic gum, 50 ml distilled water, 20 ml glycerol and 200 g chloral hydrate, followed by filtering the solution through 8-10 layers of cheesecloth or fine gauze before use (Ash & Orihel 1991).

Study of soft internal structures

To study soft parts, the monogeneans should be studied following staining. Different stains, mostly carmine-based, are used to visualise the internal structures and organs of monogeneans, *e.g.*, iron acetocarmine (Georgiev *et al.* 1986; see the procedure below), Schneider's acetocarmine, Mayer's acid carmalum, Gomori's trichrome, etc. (see also Humason 1979; Ash & Orihel 1991 for more details on several staining techniques). After staining, the worms are dehydrated in ascending series (increasing concentration) of ethanol, cleared (with clove oil or xylene), and finally mounted in Canada balsam as permanent preparations, which are suitable for long-term storage in museum collections.

PROCEDURE

1. Prior to staining, rinse the fixed worms in distilled water (30-60 min); worms fixed/preserved in 70% ethanol can be stained directly, without previous rinsing.
2. Transfer the worms to iron acetocarmine in a small Petri dish and keep them in the staining solution until they acquire a deep red colour (1-10 hours).
3. Rinse the worms by placing them into 70% ethanol.
4. Destain the worms in a weak solution of acid ethanol (1 ml or 4 drops of concentrated hydrochloric acid in 100 ml of 70% ethanol); leach the colour from the worms until they turn into a pale pink whereas the internal organs remain red-coloured. Destaining may take from several minutes to several hours, but it must be observed carefully to avoid excessive destaining. If too much stain is removed, rinse the specimens in 70% ethanol and return them to the stain (*i.e.*, start again with step 2), otherwise continue with step 5.
5. Rinse the worms by placing them into tap water until they turn into a deep red colour.
6. Dehydrate the worms through 70% (5 min), 96% (10 min) and 100% ethanol (5 min).
7. Clear the worms in clove oil (eugenol) for 5 min.
8. Mount the worms in Canada balsam as permanent slides.

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3.3.4. ENDOPARASITIC HELMINTHS

Tomáš SCHOLZ, Roman KUCHTA, Šárka MAŠOVÁ & Aneta KOSTADINOVA

Introduction

In the present text, general study methods are briefly described for different groups of endoparasitic helminths, *i.e.*, adults and larvae of flukes (Trematoda, *i.e.*, Aspidogastrea and Digenea), tapeworms (Cestoda), parasitic nematodes (Nematoda) and spiny- or thorny-headed worms (Acanthocephala). The main focus of this section is on the most important steps in searching for endohelminths and on their fixation and processing to ensure adequate quality of the material collected for subsequent evaluation. More detailed information can be found in specialised papers or books on individual groups of endoparasitic helminths.

Examination of fishes for endoparasitic helminths

Parasitological (helminthological) dissection is the basic method to obtain parasites. The extent of the examination depends on the objectives of a given study. The present text is focused on endoparasitic helminths and thus only the examination of internal organs will be described. As mentioned in the introduction to this methodological section (see 3.2), it is necessary to examine fresh hosts because worms, especially tapeworms and tiny trematodes, die quickly following the host's death. As a result, endoparasitic worms from long-time dead or frozen hosts are decomposed and unsuitable for subsequent studies including their reliable identification. Data on the host identity, site of infection, number of specimens found and fixed, fixative used, the date of dissection and the name of the collector should be written in a field notebook. It is highly recommended to record this information digitally on spreadsheets (such as Excel files) following fieldwork.

PROCEDURE (Fig. 3.3.4.1)

1. Take (a) photograph(s) of the host to be examined (the head of the fish should be on the left side) with its unique code (see below) and measurements (usually total and standard lengths). It is strongly recommended to excise a small piece of fish tissue (*e.g.*, muscle, fin – 'finclip', or liver) and fix it in molecular-grade ethanol to allow DNA-based identification of the host or other genetic work on the hosts, *e.g.*, barcoding, co-phylogenetic work, etc.

2. Place the complete digestive tract and other internal organs either in a suitable Petri dish or on a glass plate and add a small volume of saline (0.8-0.9% physiological solution, *i.e.*, 8-9 g of NaCl in 1 l of water). Under no circumstances should the organs dry out. In the tropics, you can add small pieces of ice to the Petri dish with the organs to cool the saline and thus slow down the decomposition of organs and parasites. Add labels with a unique host