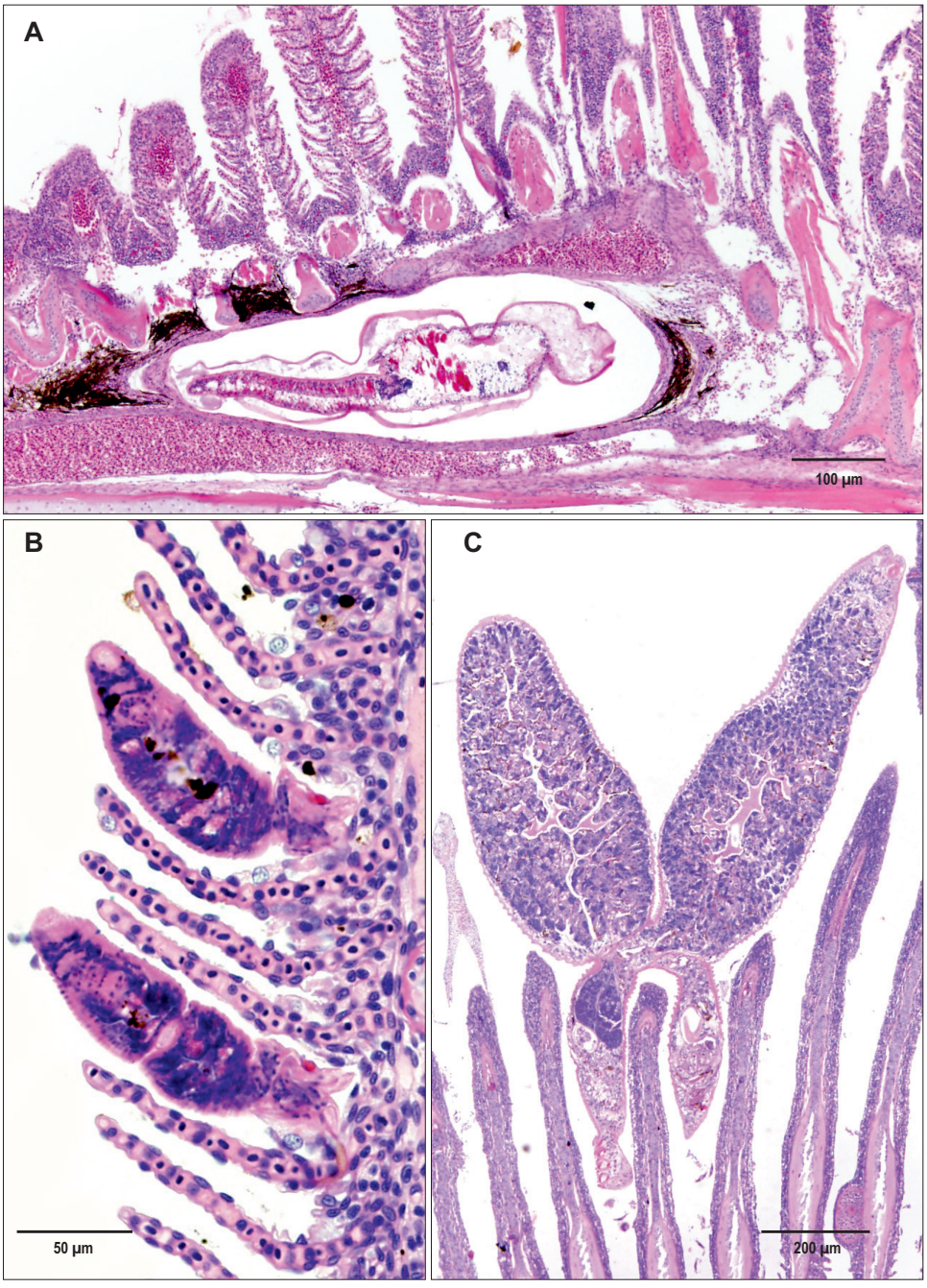

Fig. 3.4.3. A. Metacercarial stage of a digenean (Trematoda) in the gill arch of *Cichlasoma* sp.; **B.** Longitudinal sections of dactylogyrid monogeneans among secondary gill lamellae of *Haplochromis* sp., attached to epithelial tissue of gill filament; **C.** Complete section of a polyopisthocotylid monogenean *Diplozoon* sp. among gill filaments exemplifies the potential of histology in parasite identification. All H & E.

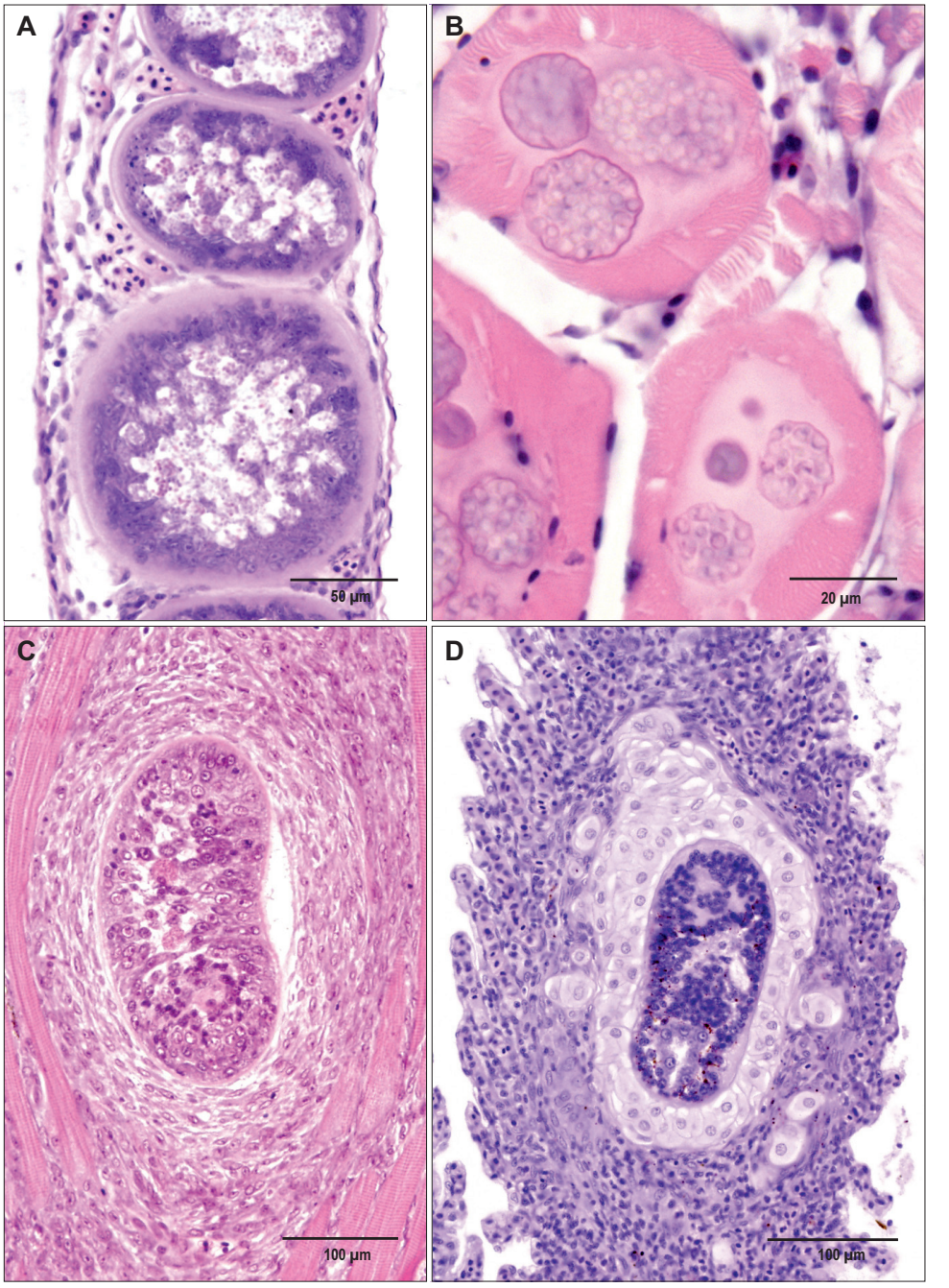

Fig. 3.4.4. A. Xenoma formations induced by microsporidia in connective tissue of *Nothobranchius* sp. Spores concentrated in the centre are almost indistinguishable whereas the whole formation can reach macroscopically visible size; **B.** Developmental stages of microsporidia in muscle fibres of *Paracheirodon innesi*; **C.** Metacercaria of a digenean trematode in muscle tissue of *Haplochromis* sp.; **D.** Metacercaria in cartilage of gill filament. All H & E.

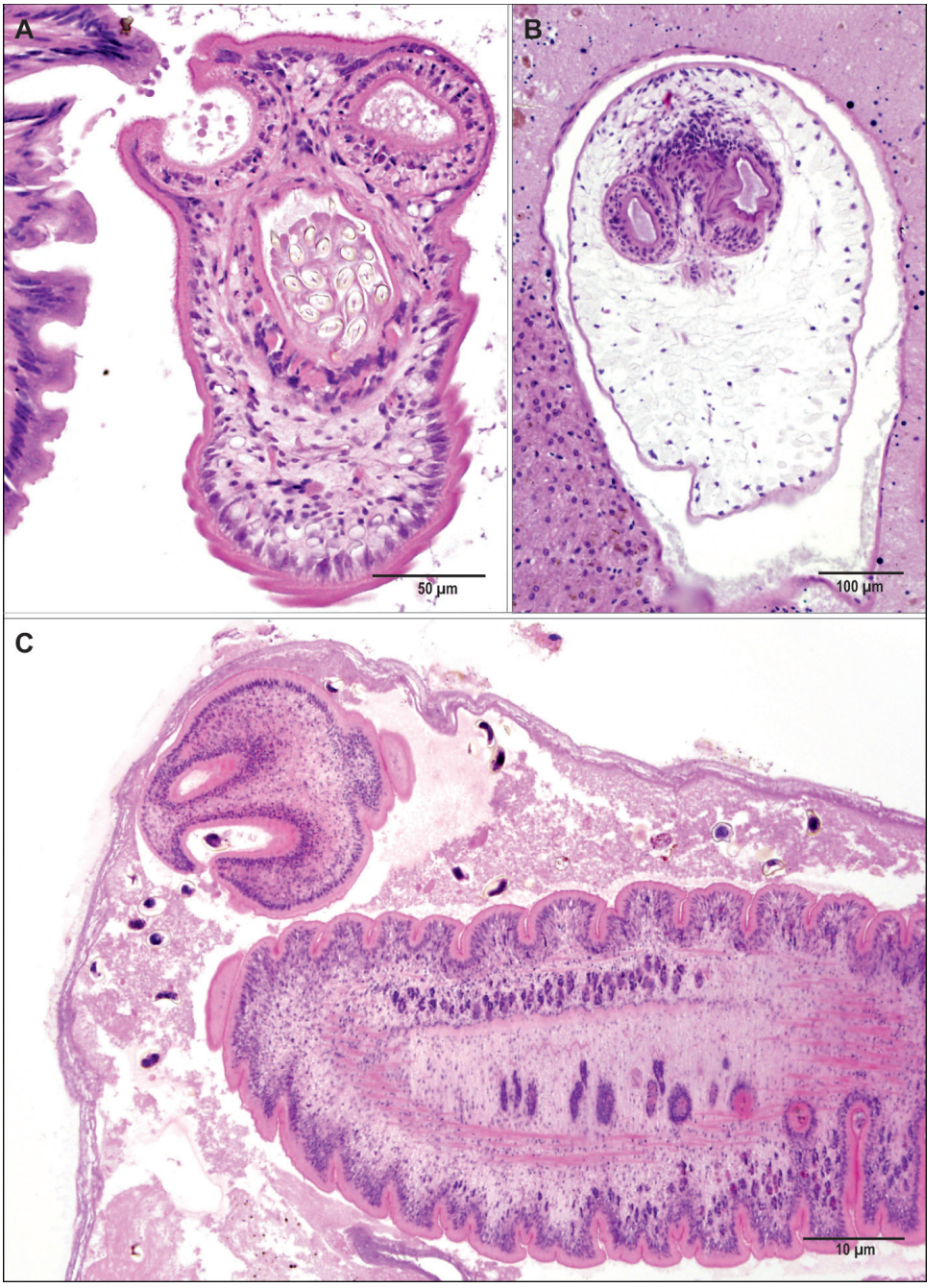

Fig. 3.4.5. A. A sucker-bearing gryporhynchid cestode with hooks on the rostellum in the intestine of *Sandelia* sp.; **B.** Larval stage (plerocercus) of a gryporhynchid cestode in the liver of a cichlid fish; **C.** Cestode *Schyzocotyle acheilognathi* (Yamaguti, 1934) with a pair of dorsoventral grooves (bothria), part of neck and a short part of the strobila in the intestine of *Symphysodon* sp. The fish tissue is autolytic whereas the structures of cestode are well maintained. All H & E.

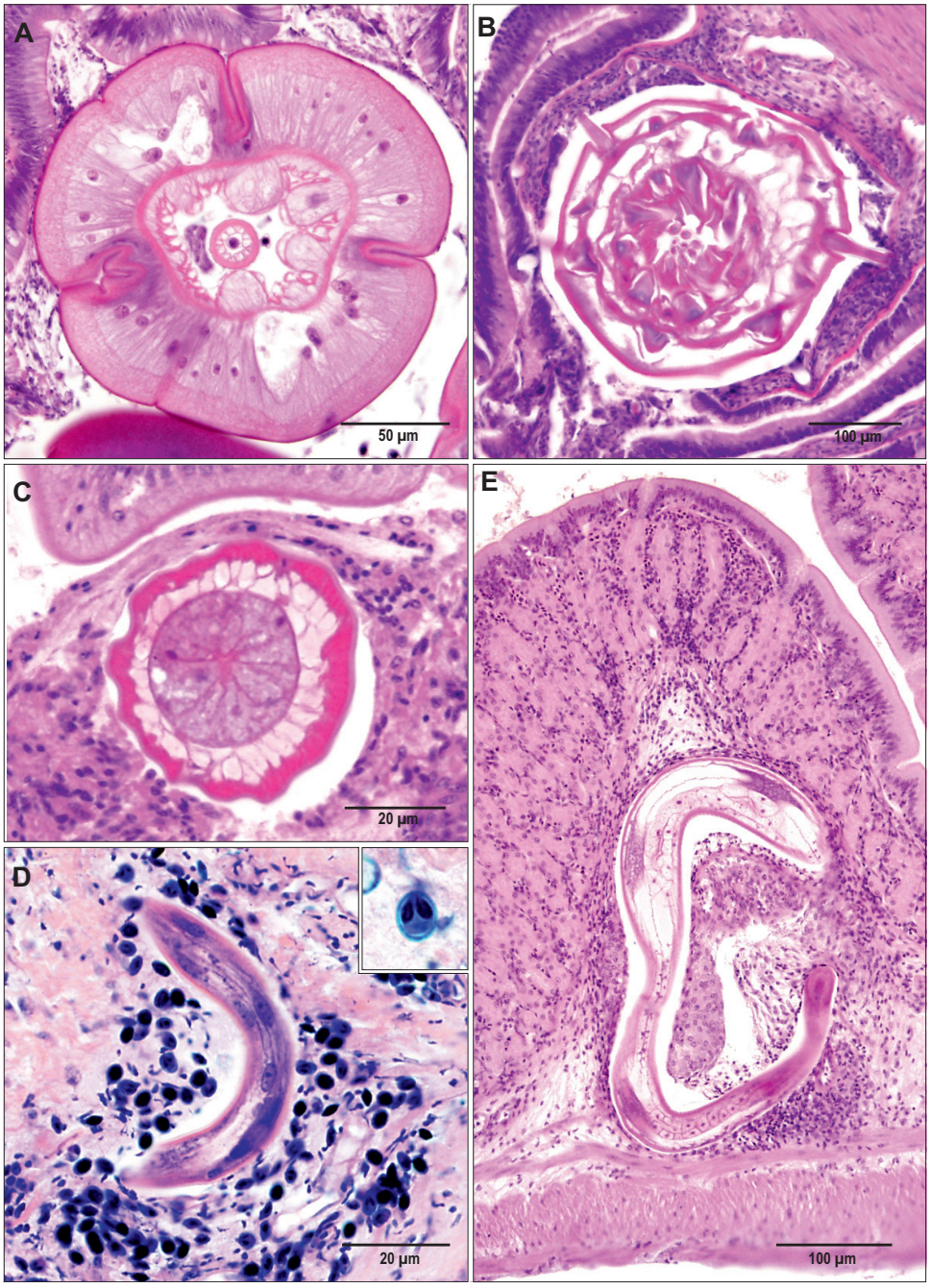

Fig. 3.4.6. A. Transverse section of the anterior part of an acanthocephalan. H & E; **B.** Transverse section of everted acanthocephalan proboscis armed with hooks. H & E; **C.** Transverse section through the oesophagus of a nematode. H & E; **D.** Larval stage of a nematode in host connective tissue. Also note the darkly stained myxospores (inset). Giemsa stain; **E.** Larval stage of a nematode in connective tissue of the stomach. H & E.

Fixation

The objectives of fixation are to preserve tissue samples, preventing autolysis and putrefaction. Fixation has to be adequate and complete. These requirements determine the type of fixative and the method of fixation applied. There are routinely used fixatives which fix tissue samples relatively slowly (*e.g.*, neutral buffered formalin solution), aggressive, rapidly penetrating fixatives (*e.g.*, mixtures of formol, acetic acid and ethanol), and fixatives which preserve specific cell components for specific staining procedures (*e.g.*, non-aqueous fixatives for glycogen). If a fixative causes tissue distortions and deformities, it is recommended to trim tissue samples before the next step (dehydration) starts.

Dehydration

To avoid excessive shrinkage of tissue samples, which ultimately causes difficulties in the evaluation of lesions, water should be eliminated from the samples almost completely by using ascending grades of ethanol before being transferred into an organic solvent. The best results are obtained with adequate concentrations of ethanol and adequate exposure times to ethanol and organic solvent.

Embedding

Embedding following dehydration consists of gradual impregnation of tissue samples with a firm medium (paraffin with a melting point of 56.6°C, mixtures of paraffin with other components, etc.) and blocking out in appropriate moulds. Of the considerations that should be kept in mind in the three-step impregnation procedure (three baths of paraffin), the most important ones are to follow the impregnation schedules given for each paraffin to eliminate remnants of organic solvents (xylene, toluene, etc.) completely. Too long exposures in paraffin and/or the presence of solvents in the last paraffin bath impair the quality of blocks and sections. The paraffin-impregnated tissue should be oriented with the side of interest facing the bottom of the mould.

Sectioning

The essential equipment required for sectioning properly prepared tissue blocks includes a microtome adjusted for the type of knife used, a water bath, slides and a hot plate (or a safe place to dry sections). To ensure good results from the sectioning, several adjustments may prove necessary. Of those, the crucial one is an appropriate knife angle as specified by the manufacturer. Also important are the temperatures of the water bath, hot plate and oven (with paraffin, with its melting point 56.6°C, neither of these temperatures should exceed 45°C) and drying the sections completely.

Staining of histological sections

The staining procedure completes the preparation of tissue material for histological examination. It includes deparaffinisation of sections with xylene or another organic solvent, their hydration to water (through descending concentrations of ethanol), staining proper and counterstaining, followed by dehydration (through an ascending series of ethanol), clearing (with xylene) and mounting in a medium of choice. It is advisable to have a sufficient number of consecutive sections in order to avoid missing important details which might require special staining.

Haematoxylin and eosin are universally accepted basic dyes used to demonstrate tissue morphology. Haematoxylin stains the nuclear material whereas eosin stains the cell cytoplasm. Some special methods also deserve to be mentioned here, *e.g.*, the Periodic Acid Schiff reaction for demonstration of mucopolysacharides, Trichrom methods with various counterstaining agents for connective tissue, Van Gieson's method for collagen, Giemsa staining for protozoans, Gram's stain for Gram positive and Gram negative bacteria, Ziehl-Nielsen for acid alcohol fast organisms, Von Kóssas for demonstration of calcium salts, Perl's method for iron, Gomori for fungi, etc. There are many methods elaborated by specialists and many modifications of individual techniques. One can find dozens of recipes or modifications of basic staining methods but hardly ever a clear explanation of the chemical processes taking place during the staining. More than 50 staining procedures have been modified for microwave technology, saving time and liquids. For more detailed information, see list of references below.

References

Bancroft, J.D. & Stevens, A. 1975. *Histopathological Stains and Their Diagnostic Uses*. Churchill Livingstone, Edinburgh: 149 pp.

Bancroft, J.D. & Cook, H.C. 1984. *Manual of Histological Techniques*. Churchill Livingstone, Edinburgh: 274 pp.

Bancroft, J.D. 2008. *Theory and Practice of Histological Techniques*. Elsevier Health Sciences, Philadelphia: 725 pp.

Boon, M.E. & Kok, L.P. 1989. *Microwave Cookbook of Pathology. The Art of Microscopic Visualization*. Second Edition. Coulomb Press, Leyden: 224 pp.

Bruno, D.W., Nowak, B. & Elliot, D.G. 2006. Guide to the identification of fish protozoan and metazoan parasites in stained tissue sections*. Diseases of Aquatic Organisms* 70: 1-36.

Dyková, I. & Lom, J. 2007. *Histopathology of Protistan and Myxozoan Infections in Fishes: An Atlas*. Academia, Prague: 219 pp.

Ferguson, H.W. 2006. *Systemic Pathology of Fish: A Text and Atlas of Normal Tissues in Teleosts and Their Responses in Disease*. Second Edition. Scotian Press, London: 368 pp.

Grizzle, J.M. & Rogers, W.A. 1976. *Anatomy and Histology of the Channel Catfish*. Auburn Printing Inc., Auburn: 99 pp.

Hewitson, T.D. & Darby, I.A. 2010. *Histology Protocols*. Series: Methods in Molecular Biology, Vol. 611. Springer, Berlin: 230 pp.

Howard, D.W., Lewis, E.J., Keller, B.J. & Smith, C.S. 2004. *Histological Techniques for Marine Bivalve Molluscs and Crustaceans*. NOAA Technial Memorandum NOS NCCOS 5. U.S. Department of Commerce: 218 pp.

Hybiya, T. (Ed.) 1982. *An Atlas of Fish Histology, Normal and Pathological Features*. Kodanska Ltd., Tokyo: 147 pp.

Lillie, R.D. 1965. *Histopathologic Technique and Practical Histochemistry*. Third Edition. McGraw-Hill, New York: 715 pp.

Luna, L.G. (Ed.) 1968. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. McGraw-Hill, New York: 258 pp.

Luna, L.G. 1992. *Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts*. American Histolabs, Inc., Gaithersburg: 767 pp.

Presnell, J.K. & Schreibman, M.P. 1997. *Humason's Animal Tissue Techniques*. Fifth Edition. Johns Hopkins University Press, Baltimore: 572 pp.

Yasutake, W.T. & Wales, J.H. 1983. *Microscopic Anatomy of Salmonids: an Atlas*. U.S. Department of the Interior, Fish and Wildlife Service, no. 150: 189 pp.

Chapter 3.5.

MOLECULAR TAXONOMY

Ivan FIALA & Jan BRABEC

Introduction

Deoxyribonucleic acid (DNA) sequences are a valuable source of information that stores the elementary instructions for how individual parts of an organism should be assembled and operate. DNA-encoded information can also be used to gain insights into the evolutionary history of an organism. Recovering this information has become an essential strategy to study and compare organisms. The field of downstream computational molecular evolution approaches has grown into a complex and rapidly evolving scientific discipline.

Analyses of DNA sequences have become an important part of various studies on the parasites of fish globally, including alpha taxonomy, diagnostics of disease agents, phylogeographical distribution and various studies on the biology of parasites including ecological, life cycle or host specificity-oriented surveys, to name a few. In strong contrast to that, the use of molecular data in studies on African fish parasites remains limited. Studies of Pouyaud *et al.* (2006), de Chambrier *et al.* (2008), Kuchta *et al.* (2012) and Přikrylová *et al.* (2013) are among the few available examples, where analyses of gene sequences assisted substantially in resolving the phylogenetic position of various fish helminths from the African continent. Schaeffner *et al.* (2011) and Chibwana *et al.* (2013) used molecular data to study phylogenetic relationships within individual genera of fish tapeworms and trematodes, respectively.

Co-phylogenetic analyses allowed Mendlová *et al.* (2012) and Vanhove *et al.* (2015) to propose speciation mechanisms in monogeneans infecting African cichlid fishes. Bouzid *et al.* (2013) studied genetic divergence within populations of the diphyllobothriidean cestode *Ligula intestinalis* (Linnaeus, 1758) using highly variable sequences of non-coding regions of DNA, whereas Kmentová *et al.* (2016) used sequence data from the nuclear ribosomal DNA region and the cytochrome *c* oxidase subunit I gene to look at, respectively, host range and intraspecific diversity in the dactylogyrid monogenean *Cichlidogyrus casuarinus* Pariselle, Muterezi Bukinga et Vanhove, 2015. Brabec *et al.* (2016) used next generation sequencing to study intraspecific differences within isolates of the invasive Asian fish tapeworm *Schyzocotyle acheilognathi* (Yamaguti, 1934) parasitising African fishes. Additionally, sequence data are frequently used in species descriptions to support the identification or discovery of parasite species, or to get an idea of their phylogenetic position.

Among the molecular markers most frequently used to study phylogenetic relationships and life history characteristics of fish parasites are ribosomal RNA (rRNA) encoding genetic loci, which include three rRNA encoding genes called 18S, 5.8S and 28S rDNA, according to their molecular weight, as well as the noncoding sequences of so-called internal transcribed spacers 1 and 2 (ITS-1, ITS-2) that are situated between 18S and 5.8S, and 5.8S and 28S rDNAs, respectively. Individual coding and noncoding regions from this genetic locus together form a unit called the nuclear rRNA operon, which is typically found in several hundreds to thousands of copies that are tandemly repeated one unit after another on certain chromosomes, depending on the parasite group. A wealth of information on molecular characteristics of rDNA has been summarised by Blair (2006) for parasitic flatworms and by Fiala *et al.* (2015) for myxozoan parasites.

Given the heterogenetic nature of individual parts of the nuclear rRNA operon, individual regions differ by their relative mutation rate and thus their speed of evolution. Therefore, they can be used across a range of taxonomic levels, spanning from populations of a single parasite species to orders and classes of parasites. Typically, the noncoding regions (such as ITS regions) are used at lower taxonomic levels (*i.e.*, populations and species), whereas the gene sequences (18S, 28S rDNA) are useful at higher levels, typically from genera to orders. However, for many parasitic groups, *e.g.*, Myxozoa or ciliates of the family Trichodinidae, 18S rDNA is a standard universal marker from species to order levels (Tang *et al.* 2013; Fiala *et al.* 2015). Combination of 18S and 28S rDNA or both noncoding and coding regions can be used in studying the phylogenetic relationships of parasites (*e.g.*, Bartošová *et al.* 2009; Přikrylová *et al.* 2017). Thanks to the presence of relatively conserved regions, rRNA loci can be characterised using a universal set of short strands of nucleotides called primers that are necessary to amplify a given region of DNA during polymerase chain reaction (PCR).

Sequences of mitochondrial protein-coding and rRNA-encoding genes (mitochondria are remnants of a primary endosymbiotic event and thus carry their own pair of rRNA genes originally belonging to an alpha proteobacterium) are further examples of commonly used molecular tools. Contrary to the nuclear rDNA, their overall speed of evolution tends to be higher (in some cases roughly comparable to ITS regions of the nuclear rRNA operon). This makes mitochondrial genes useful candidates for lower-level taxonomical studies. However, they may also be used on higher taxonomic ranks, when the protein-coding nucleotide sequences are translated into the corresponding sequence of amino acids. However, the increased mutation rate also means that universal primers are difficult to design. Moreover, flatworms substantially differ from other metazoans in amino acid content over cytochrome c oxidase I, *i.e.*, the sequence homology of flatworm and other metazoans' *cox*1 sequences is generally lower than sequence homology within metazoan *cox*1 (Vanhove *et al.* 2013) and researchers are thus left with no other option than to design a specific set of primers for their parasitic group of interests.

Recently, next generation sequencing techniques have been developed and gained popularity to bulk-characterise sequence data on large scales (*i.e.*, from thousands of loci to entire genomes) without previous knowledge of primer sequences. However, these sequencing approaches remain expensive and require the use of sophisticated technologies and highly trained laboratory staff and bioinformaticians, and are thus not suitable for routine taxonomy.

Fixation of fish parasites for molecular studies

The most critical step that allows successful isolation of DNA and generation of sequence data is quick and correct processing of the dissected parasite tissue and its immediate preservation in a suitable preservative. As a rule of thumb, parasites should be processed after their isolation from the host without any time delays, preferably immediately after the host's death. Extracted parasites (or infected tissues) should either be immediately preserved or kept in conditions that allow parasite survival (*i.e.*, in cool temperatures, appropriate pH and salt concentration). Before being completely submerged in the appropriate preservative (see Chapter 3.3), cells of parasitic protists or tissues of metazoan parasites have to be carefully cleaned of any remnants of the host cells and tissues, eliminating carry-over and subsequent simultaneous extraction of host DNA. Nearly absolute (96-99%) molecular-grade ethanol is used as a preservative of choice, notably in hot weather climate conditions.

DNA sequencing

Sequencing of selected molecular markers includes several steps (principally DNA isolation, PCR amplification and electrophoresis), which require adequate equipment and laboratory experience. A number of essential laboratory skills need to be acquired first to ensure successful and safe work in the laboratory. A good start for those not familiar with basic laboratory practice is to get familiar with individual chapters of the Current Protocols Essential Laboratory Techniques (http://onlinelibrary.wiley.com/book/10.1002/9780470089941) and preferably to obtain practical skills personally in an established laboratory under the supervision of a technician experienced in all relevant methods. Most of these complex issues can be eased through collaboration with an expert parasitologist with a publication record that includes the use of molecular taxonomy and phylogenetic approaches.

DNA isolation

The first step in the entire process of characterising novel sequences is isolation and purification of the DNA from the cells, the basal building blocks of any parasite's body. Within the cells, the DNA is located in membrane-bound organelles, where it is part of high-molecular complexes that consist of DNA itself together with a number of associated proteins. The goal of the DNA extraction step is to get the DNA out of these cells, into a protein- and other contaminant-free water solution called a buffer. It is essential to obtain well-purified DNA in this step, otherwise the following step (*i.e.*, PCR amplification) is likely to fail.

Generally, there are two basic, frequently used ways of isolating DNA. The first involves the use of a commercial DNA extraction kit (basically a box that includes all the chemicals and silica membrane spin columns necessary for DNA isolation, commercially available from many biotech companies). The second option is to go through a more traditional procedure called phenol-chloroform extraction. Both of these methods can vary slightly from one another according to the company that manufactures the kit and the authority that originally established the actual phenol-chloroform protocol. General principles and practical descriptions of sample protocols can be found in Dowhan (2012). As an oversimplification, both DNA extraction strategies are based on the digestion of the cells or tissue and the separation of the DNA from its associated proteins in a clean, water-based buffer.

Independent of the extraction protocol, all workflows start with transferring a certain volume of parasite cells or a small piece of tissue from the ethanol preservative into a new, clean 1.5 ml Eppendorf tube. The transferred material needs to be ground into as small pieces as possible (in the case of tissue) without risking the actual loss of the tissue, especially when you possess only small snippets of, for example a tapeworm strobila (often barely seen with the naked eye). In the case of larger parasites, you should cut up to 5 mm^3 of the tissues with clean, sterilised stainless steel dissecting scissors or a blade, and after a transfer into the new tube, cut it into as small pieces as possible immediately, before the ethanol evaporates and the tissue becomes solid, hard to cut and starts moving because of static electricity. Between processing individual specimens, make sure to thoroughly clean the forceps and scissors/blade used for transferring and cutting the tissue, to avoid cross-contamination of the sample by exogenous DNA that would be impossible to discover in later steps. A recommended method of cleaning is rubbing the forceps/scissors/blade well with a sterile piece of tissue soaked with absolute ethanol, and sterilising the steel tools over a laboratory burner. The tools should be cooled down before processing the next tissue sample.

PCR (polymerase chain reaction) amplification

Polymerase chain reaction is a method to amplify, starting from the solution of parasite DNA, a selected molecular marker that will be used, *e.g.*, to reconstruct the phylogeny of the studied parasite taxon. To amplify the chosen marker (*e.g.*, 18S rDNA), the following chemicals and tools are needed: Taq DNA Polymerase, Taq Reaction Buffer, dNTPs, forward and reverse primers, PCR-grade water and DNA template; thermocycler, pipets, tubes, tips and gloves. PCR is a routine method in many molecular laboratories and detailed protocols can be found elsewhere (*e.g.*, Sambrook *et al.* 1989). For successful amplification, good quality DNA and well-designed primers are crucial.

Electrophoresis

Agarose gel electrophoresis is the most effective way of separating DNA fragments of varying sizes ranging from 25 kb to 100 bp. The phosphate backbone of the DNA (and RNA) molecule is negatively charged. Therefore, DNA fragments will migrate to the positively charged anode when placed in an electric field. Since

DNA has a uniform mass/charge ratio, DNA molecules are separated by size within an agarose gel in a pattern such that the distance travelled is proportional to their molecular weight.

Sequencing

The PCR product of the proper size must be cleaned from unused nucleotides and primers. The product is directly sequenced using a DNA sequencer if available or making use of the services of commercial DNA sequencing companies. The result is a chromatogram file with the desired sequence of nucleotides of the genetic marker.

Phylogenetic analysis

BLAST analysis

The chromatogram sequence file should be checked to confirm that the sequences obtained actually belong to the studied organism. PCR may accidentally amplify the host gene instead of the desired gene of the parasite species. This usually happens when the primers are not specific enough for the studied parasitic group. The easiest way to clarify the sequence origin is to perform a BLAST (Basic Local Alignment Search Tool) search at the web page: https://blast.ncbi.nlm.nih.gov/Blast.cgi. BLAST search of the nucleotide sequences will find the closest match with the sequences stored in GenBank.

Aligning and tree reconstruction

The phylogenetic relationships of the studied organism can be revealed by aligning the sequence obtained with a selected number of sequences downloaded from GenBank at www.ncbi.nlm.nih.gov. Such a dataset of sequences is subjected to tree reconstruction analyses using several methods of choice. The most commonly used methods are maximum likelihood, maximum parsimony and Bayesian inference.

There are plenty of phylogenetic programs that can be used for phylogenetic analysis. One of the best programs including all methods is Geneious, which is a very user-friendly programme. A trial version can be downloaded and used for a limited time (https://www.geneious.com/). Another option is to use MEGA – a free programme with very good user-friendly interface (http://www.megasoftware.net). A very useful manual for beginners called “Introduction to Walk through MEGA” can be obtained at: http://www.megasoftware.net/web_help_7/hc_introduction_to_walk_through_mega.htm

References

Bartošová, P., Fiala, I. & Hypša, V. 2009. Concatenated SSU and LSU rDNA data confirm the main evolutionary trends within myxosporeans (Myxozoa: Myxos-

porea) and provide an effective tool for their molecular phylogenetics. *Molecular Phylogenetics and Evolution* 53: 81-93.

Blair, D. 2006. Ribosomal DNA variation in parasitic flatworms. *In*: Maule, A.G. & Marks, N.J. (Eds). *Parasitic Flatworms: Molecular Biology, Biochemistry, Immunology and Physiology*. CABI, Wallingford, pp. 96-123.

Bouzid, W., Štefka, J., Bahri-Sfar, L., Beerli, P., Loot, G., Lek, S., Haddaoui, N., Hypša, V., Scholz, T., Dkhil-Abbes, T., Meddour, R. & Ben Hassine, O.K. 2013. Pathways of cryptic invasion in a fish parasite traced using coalescent analysis and epidemiological survey. *Biological Invasions* 15: 1907-1923.

Brabec, J., Kuchta, R., Scholz, T. & Littlewood, D.T.J. 2016. Paralogues of nuclear ribosomal genes conceal phylogenetic signals within the invasive Asian fish tapeworm lineage: evidence from next generation sequencing data. *International Journal for Parasitology* 46: 555-562.

De Chambrier, A., Sène, A., Mahmoud, Z., Mariaux, J. & Scholz, T. 2008. *Sandonella sandoni* (Lynsdale, 1960), an enigmatic and morphologically unique cestode parasitic in the osteoglossiform fish *Heterotis niloticus* in Africa. *Journal of Parasitology* 94: 202-211.

Chibwana, F.D., Blasco-Costa, I., Georgieva, S., Hosea, K.M., Nkwengulila, G., Scholz, T. & Kostadinova, A. 2013. A first insight into the barcodes for African diplostomids: brain parasites in *Clarias gariepinus* (Siluriformes: Clariidae). *Infection*, *Genetics and Evolution* 17: 62-70.

Dowhan, D.H. 2012. Purification and concentration of nucleic acids. *Current Protocols Essential Laboratory Techniques* 6: 5.2.1-5.2.2.

Fiala, I., Bartošová-Sojková, P. & Whipps, C.M. 2015. Chapter 5: Classification and phylogenetics of Myxozoa. *In*: Okamura, B., Gruhl, A. & Bartholomew, J.L. (Eds). *Myxozoan Evolution, Ecology and Development*. Springer, Cham, pp. 85-110.

Kmentová, N., Gelnar, M., Mendlová, M., Van Steenberge, M., Koblmüller, S. & Vanhove, M.P.M. 2016. Reduced host specificity in a parasite infecting non-littoral Lake Tanganyika cichlids evidenced by intraspecific morphological and genetic diversity. *Scientific Reports* 6: 39605.

Kuchta, R., Burianová, A., Jirků, M., De Chambrier, A., Oros, M., Brabec, J. & Scholz, T. 2012. Bothriocephalidean tapeworms (Cestoda) of freshwater fish in Africa, including erection of *Kirstenella* n. gen. and description of *Tetracampos martinae* n. sp. *Zootaxa* 3309: 1-35.

Mendlová, M., Desdevises, Y., Civáňová, K., Pariselle, A. & Šimková, A. 2012. Monogeneans of West African cichlid fish: evolution and cophylogenetic interactions. *PLoS ONE* 7: e37268.

Pouyaud, L., Desmarais, E., Deveney, M. & Pariselle, A. 2006. Phylogenetic relationships among monogenean gill parasites (Dactylogyridea, Ancyrocephalidae)