Vol. 14: 165–173, 2012
doi: 10.3354/ab00389

AQUATIC BIOLOGY
Aquat Biol

Published online January 4

Use of laser scanning confocal microscopy for morphological taxonomy and the potential for digital type specimens (e-types)

A. J. Brooker*, A. P. Shinn, J. E. Bron

Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK

ABSTRACT: The use of laser scanning confocal microscopy (LSCM) for creating taxonomic descriptions of copepods is investigated. A new technique is described, which employs a contour filter to process digital LSCM images, allowing taxonomic information to be quickly and accurately distilled into a simple illustration. LSCM allows the imaging of whole specimens, which can be rotated and viewed from any angle—a major benefit over light microscopy. Using this technique, it is suggested that taxonomic descriptions can be rapidly produced in a fraction of the time required to produce similar descriptions using traditional light microscopy and hand drawing techniques. Good staining of specimens is, however, essential to produce accurate descriptions and more research is required in this area. The use of LSCM for morphological taxonomy shows great potential, not only for producing taxonomic descriptions, but also providing a complementary adjunct to traditional type specimens in the form of 3D digital 'e-types' deposited in recognised international databases.

KEY WORDS: E-type · Digital taxonomy · Confocal

INTRODUCTION

Taxonomic descriptions of copepods usually rely upon traditional light microscopy, supplemented by other techniques such as scanning and transmission electron microscopy where possible. Creating taxonomic drawings using light microscopy is a long and laborious process, requiring considerable expertise and often involving the careful dissection of specimens and the need to synthesise specimen drawings from many observations taken at different focal depths and using photos or a camera lucida. Laser scanning confocal microscopy (LSCM) allows whole specimens to be imaged in focus by generating image 'stacks', which can then be combined to create a 3D representation of the specimen, or compressed into a single composite image. Using LSCM the specimen is scanned point-by-point in X, Y and Z axes to give images comprising only the best focused light and rejecting out of focus/scattered light emanating from elsewhere in the specimen. This decreases noise and greatly increases the clarity and potential resolution of the final image and downstream reconstruction. Visualisation of 3D structures has been demonstrated by Klaus et al. (2003), who reconstructed the 3D aspects of insect exoskeletons, and Galli et al. (2006), who imaged monogenean sclerites in 3 dimensions, contributing significantly to our knowledge regarding the shape and functionality of these structures. In the present study, the possibility of using a single LSCM composite image to rapidly generate a 2D taxonomic drawing was investigated, using free-swimming juvenile specimens of the pennellid copepod parasite *Lernaeocera branchialis* (L., 1767). The results are compared to corresponding images from light microscopy and the benefits and potential problems of this technique are highlighted. In addition, the advantages of using LSCM-generated images for morphological taxonomy are discussed.

*Email: ajb3@stir.ac.uk

MATERIALS AND METHODS

Whiting *Merlangius merlangus* (L.) and, to a lesser degree, cod *Gadus morhua* (L.) infected with *Lernaeocera branchialis* were sampled from the catches of commercial demersal trawlers working within the River Forth Estuary at Kincardine, Scotland (56° 02′ 53′ N, 3° 40′ 59′ W). To culture the juvenile stages of *L. branchialis,* egg strings were dissected from gravid female parasites collected from infected whiting and cod. These were then maintained under aeration in 500 ml beakers kept in a WTB Binder Labortechnik precision environmental chamber at 10°C. Once the eggs began to hatch they were placed in a beaker of fresh aerated sea water (35 ppt) and after a period of time (20 min to 24 h), the egg strings were transferred to another beaker of fresh sea water, leaving behind a 'batch' of nauplii. These batches were then used for experiments as either nauplii or copepodids, depending on how long they were maintained.

Lernaeocera branchialis specimens for traditional light microscopy were prepared by clearing individuals in 85% lactic acid (Sigma L1250) for 30 min, which also softened the connective tissues, and then by dissecting them on a cavity slide using fine mounted needles under a dissecting microscope. Individual appendages from each specimen were mounted in 100% glycerol (Sigma G7757) and then sealed with clear nail varnish once a coverslip had been placed over them. Both whole and dissected specimens were viewed on an Olympus BX51 compound microscope and digital micrographs were taken using a Zeiss AxioCam MRc camera and MRGrab 1.0.0.4 software (Carl Zeiss Vision, 2001), these being used as the basis for initial drawings.

Specimens for LSCM were prepared by fixing them in 2.5% glutaraldehyde (>1 wk to enhance autofluorescence), and then rinsing them in distilled water when required. They were then stained with either Blankophor (150 µl in a watch glass filled with 5 ml distilled water for 2 h) (ICN Biomedicals) or Gomori's trichrome (Gomori 1950) overnight before rinsing again with distilled water. Individual specimens were then placed into a 35 mm glass base dish (Iwaki) and covered with distilled water before being imaged on a Leica TCS SP2 AOBS laser scanning confocal microscope coupled to an inverted Leica DMIRE2 microscope equipped with a HC PL APO 20× objective. The samples were imaged using 2 laser excitation lines (UV diode laser 405 nm and argon laser 488 nm) with fluorescence emission collected at 411 to 483 nm and 498 to 587 nm. Using the Leica Confocal Software v6.21, 8-bit TIFF images were obtained, with an image size of 1024 × 1024, 2048 × 2048 or 2048 × 1024 pixels, depending on the size of the specimen. Image stacks comprising between 138 and 280 images were scanned with an automatically optimised section thickness to maximise resolution (range 0.5 to 1.34 µm).

Specimen outlines were generated from LSCM composite images in Adobe Photoshop CS3 v.10.0 (Adobe Systems, 2007) according to the following procedure:

(1) An outline of the specimen was created using the trace contour filter and the level adjusted to pick up the specimen edges (see Fig. 1b).

(2) The outline was overlaid onto the original image (see Fig. 1c).

(3) Segmental boundaries and other features such as spines that were not contrasted against the background were drawn manually, using the original image as a guide (see Fig. 1d).

(4) The line image was desaturated and the contrast increased to make the lines appear black (see Fig. 1e).

RESULTS

The primary purpose of a taxonomic drawing is to clearly and accurately illustrate anatomical features. In this study, by using a contour filter on a LSCM composite image and manually drawing segmental boundaries, the anatomical features of the specimen were effectively distilled from the original image. Capturing sequential layers through the specimen and combining them using computer software allowed an image to be created that was in sharp focus throughout the whole specimen (Fig. 1a). The outline of the specimen was automatically generated from a whole specimen, resulting in low error, and ensuring that the shape and size of the features were accurate (Fig. 1b). By overlaying the specimen outline onto the original LSCM image (Fig. 1c), the segmental boundaries were also accurately drawn, using the LSCM image as a guide (Fig. 1d).

A major difficulty found in viewing whole specimens occurred when some features were obscured by other structures. One method of overcoming this problem, when features were on the underlying face of an appendage, was to rotate the image stack to view features from a different angle. In Fig. 2, the median setules (2 and 3 in Fig. 2c) and terminal setule (5 in Fig. 2c) of the nauplius I antennule are visible because the original image stack (Fig. 2a) was rotated to bring them into view from behind the

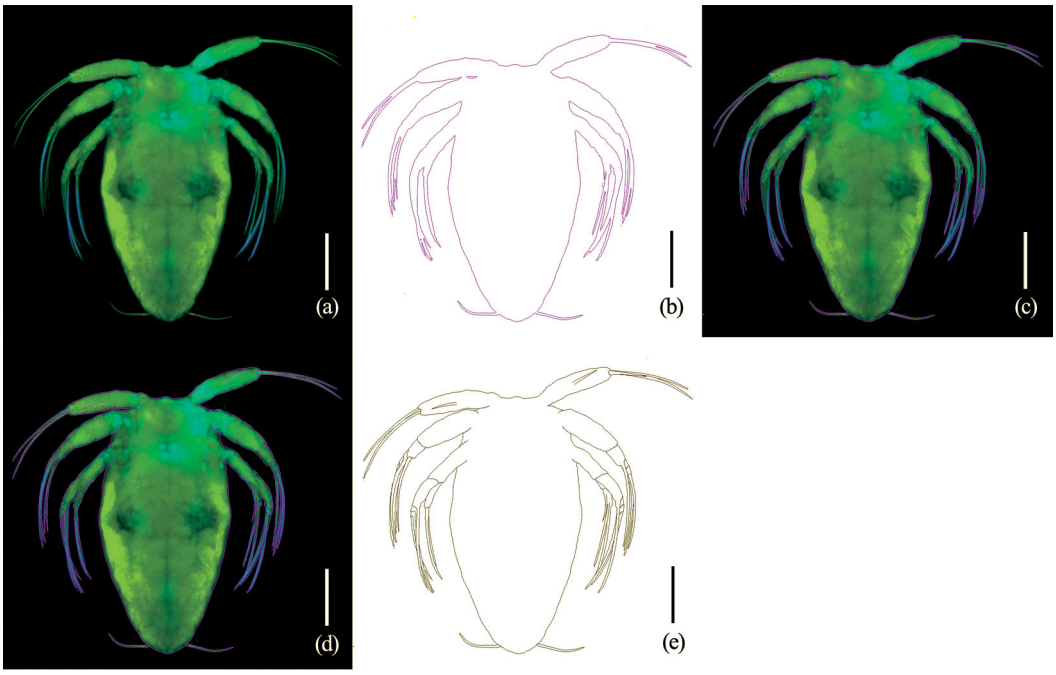

Fig. 1. *Lernaeocera branchialis* (L., 1767). Generation of a taxonomic drawing from a LSCM composite image. (a) LSCM composite image of nauplius I fixed in 2.5% glutaraldehyde and stained with Blankophor; (b) automatically extracted outline; (c) outline overlaid on original image; (d) segmental boundaries added to outline; and (e) outline desaturated and contrast increased. Scale bars: 50 µm

endopod (Fig. 2b). Rotating the LSCM image stack also allowed appendages from whole specimens that were not situated along the same plane as the focal plane to be viewed flat, minimising deformation and reducing errors. In Fig. 3, the LSCM image stack was rotated to view the maxilla of the copepodid along the flat plane (Fig. 3a), showing that the basal ramus is wider and the terminal ramus is longer (Fig. 3b) than shown in the corresponding light microscope illustration (Fig. 3c), where a non-planar orientation resulted in an underestimation of the width of the appendage. As the maxilla is too small to be dissected by hand and flattened on a slide it was not possible to view it on the flat plane using light microscopy. Although rotating the image stack does allow these smaller features to be viewed from different angles, it does result in a loss of clarity. It was found that even if the vertical resolution (i.e. thickness of each image plane) was optimised, the edges of features can appear fuzzy once rotated and more noise is present in the image (Fig. 3a), sometimes making it difficult to use a contour filter to create an outline and requiring the appendage outline to be drawn manually. This may, however, be overcome in many instances by creating higher resolution images (more pixels per micron) when scanning, taking more sections and, where necessary, using digital deconvolution techniques, to improve image clarity. Such steps were not, however, found to be necessary in the current study.

The problem of features being obscured by overlying structures was also apparent in the swimming legs of the copepodid (Fig. 4). As the swimming legs overlaid each other, the setae could not be separated by the contour filter and the protopod of the second swimming leg was hidden by the first swimming leg (Fig. 4a). Due to the positioning of the swimming legs on the body it was not possible to view them separately without dissecting individual legs. However, this would be a difficult and time-consuming process, and may result in distortion of the appendage. It would also negate the advantage of rapid production of taxonomic drawings using LSCM, which is one of its major advantages over light microscopy. A better solution was found by creating a series of composite images from the image stack, each generated from a portion of the entire stack (Fig. 4b). In each individual composite image, the visible portions of the appendages were separate from each other, allowing the unwanted appendage to be erased manually. The edited images could then be overlaid to create a final image of the desired appendage removed from the surrounding structures (Fig. 4c). This technique was also used to image the copepodid antennule in Fig. 5.

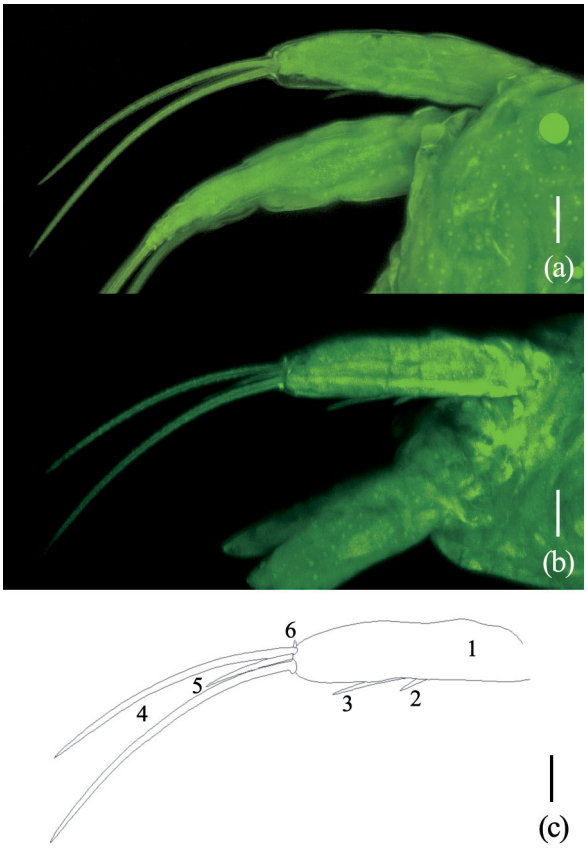

Fig. 2. *Lernaeocera branchialis* (L., 1767). Rotation of an image stack to view obscured features. (a) LSCM composite image of nauplius I antennule fixed in 2.5% glutaraldehyde and stained with Blankophor; (b) image stack rotated to view previously obscured setules; (c) resultant line drawing. Scale bars: 25 µm. Labels: 1, endopod; 2, distal median setule; 3, proximal median setule; 4, plumose setae (hairs omitted); 5, terminal setule; 6, terminal spine

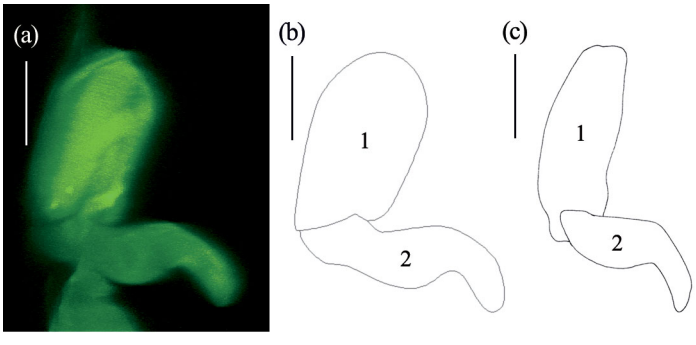

Fig. 3. *Lernaeocera branchialis* (L., 1767). Rotation of an image stack to eliminate distortion. (a) LSCM composite image of copepodid maxilla fixed in 2.5% glutaraldehyde, stained with Blankophor and rotated to view on the flat plane; (b) resultant line drawing; (c) line drawing derived from light microscope images showing how a non-planar orientation can result in distortion of the appendage. Scale bars: 25 µm. Labels: 1, basal ramus; 2, terminal ramus

A further consideration when creating taxonomic drawings is the time and training required to create such images. In this study using LSCM, once specimens had been stained, a set of images for a single specimen could be generated in under an hour. In comparison, dissecting small specimens and making drawings using a series of photographs at different focal depths was a lengthy process and one that required considerable skill and training to both manipulate the image and correctly recognise and draw the anatomical features of the specimen. With LSCM, a single image may be created that is in sharp focus throughout the whole specimen, allowing a taxonomic drawing to be made from one image. The fluorescence of the specimen also contrasts against the dark background of the image, which made it easier to delineate the outline using a contour filter. Although the initial drawing process is rapid, further time and greater taxonomic expertise were required to accurately interpret and draw the segmental boundaries, this needing greater knowledge of the anatomy of the group being studied.

Quantitative measurements are an important element of taxonomic description, and in this study, measurements were made using both light microscopy and LSCM. The ability to rotate 3D images to give a planar view using LSCM and the archiving of size data with each image (this latter also being possible with digital light microscopy imaging), allows highly accurate measurements to be made instantly on screen. With this technique, there was therefore less room for the error that can occur through specimen orientation artefacts or manual measurement using the light microscopy method.

The fixing and staining of specimens for creating taxonomic drawings using LSCM is an important consideration as this determines the fluorescence and hence final morphological appearance of the specimen. Although the specimens used in this study had some autofluorescence, the use of stains or fixatives to enhance the fluorescence of the cuticle greatly improved results. For this application, the best results were achieved with Blankophor, which binds to chitin found in the cuticle of *Lernaeocera branchialis*. Both fresh and fixed specimens were used, although fixation in glutaraldehyde caused the cuticle and underlying tissue to autofluoresce strongly, so specimens fixed in 2.5% glutaraldehyde were used for the final images. In this study, the visualisation of smaller features, such as

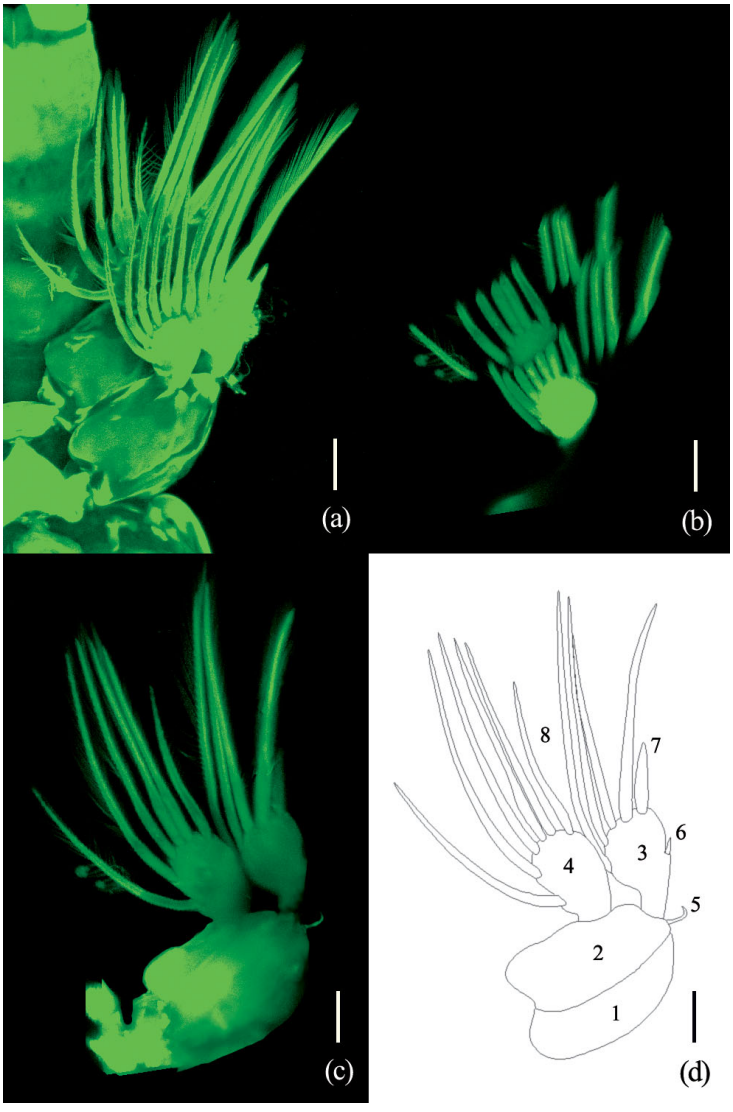

Fig. 4. *Lernaeocera branchialis* (L., 1767). Extraction of an appendage from a whole specimen. (a) LSCM composite image of copepodid fixed in 2.5% glutaraldehyde and stained with Blankophor; (b) composite image consisting of a portion of the rotated 3D image stack showing how portions of individual appendages are separated, allowing unwanted features to be erased; (c) recombined, edited portions of the 3D image stack, showing the second swimming leg extracted from the surrounding features; (d) resultant line drawing. Scale bars: 25 µm. Labels: 1, coxa; 2, basis; 3, exopodite; 4, endopodite; 5, setule; 6, spine; 7, papilliform outgrowth; 8, plumose setae (hairs omitted)

spines and setules, was sometimes problematic due to lack of fluorescence. For example, only 8 setae are visible on the terminal tip of the copepodid antennule in the LSCM image (Fig. 5a,b), whereas in the light micrograph drawing 13 setae can be seen (Fig. 5c).

Using alternative fluorescent stains, however, different features can be highlighted which would not be readily visible using light microscopy or scanning electron microscopy (SEM). Using Gomori's trichrome enabled the fine hairs on the mandible of nauplius II to be picked out by the trace contour filter, so the number of hairs on the setae could be counted (Fig. 6b). However, care must be taken when interpreting this kind of image as hairs were only visible on one side of each seta (Fig. 6a), and whilst some setae of other appendages have no hairs, in the mandible they are known to occur on both sides of all setae. Using Gomori's trichrome also enabled the small protrusions at the terminal tip of the nauplius II (likely to be developing setae of the caudal rami) to be visible extending into the body (Fig. 7), which may help to unravel the ontogeny of the caudal rami.

DISCUSSION

Taxonomic description using LSCM

The use of LSCM has several advantages over traditional methods of creating hand drawn images from light microscopy or SEM, the first and foremost being the ability to image whole specimens and rotate them as necessary to view individual features. In all types of microscopy, viewing whole specimens can be problematic where some features are obscured by other structures. This problem is often overcome by dissecting specimens and mounting structures individually on glass slides and flattening them using a cover slip. However, this study has illustrated that by using LSCM, an image stack can easily be rotated to view features from different angles. Rotating the image stack can also allow appendages from whole specimens that are not situated along the same plane as the focal plane to be viewed flat, minimising deformation and reducing errors (Galli et al. 2007).

Using light microscopy, distortion can often be a problem, depending on the method used. Specimens are often dissected to view appendages and the focus is changed to view different features, which can easily result in morphological inaccuracies in the drawing, both from distortion of soft appendages during dissection, and error whilst drawing the appendages from several images at different focal

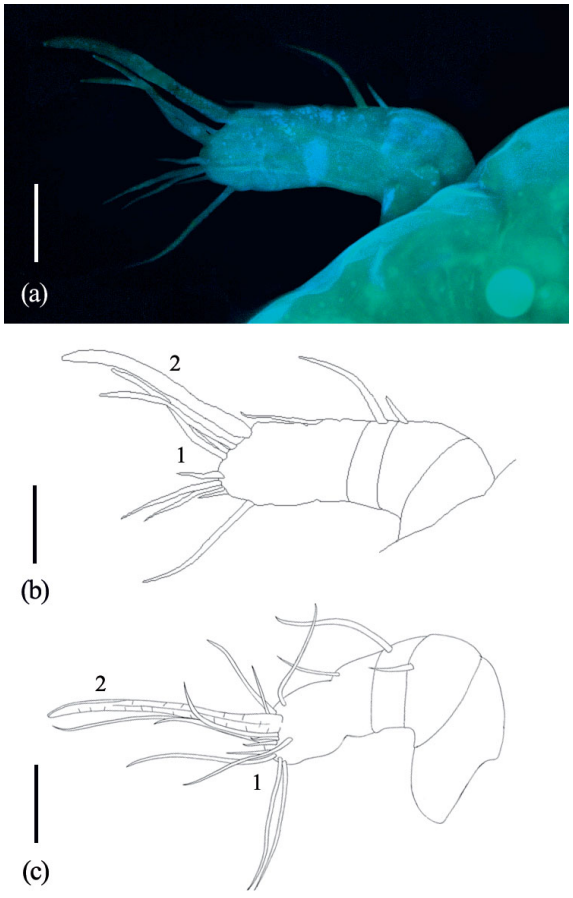

Fig. 5. *Lernaeocera branchialis* (L., 1767). Problems associated with staining of specimens. (a) LSCM composite image of copepodid antennule fixed in 2.5% glutaraldehyde and stained with Blankophor; (b) resultant line drawing showing 8 setae on the terminal tip (5 missing); (c) line drawing derived from light microscope images showing 13 setae on the terminal tip. Scale bars: 25 µm. Labels: 1, setae; 2, aesthetasc

depths. Specimens are often compressed on a slide using a coverslip, to allow the whole specimen to be viewed, which can also distort the specimen and result in inaccurate drawings (Galli et al. 2007). Where whole specimens are imaged suspended in a droplet, distortion can be a problem as a result of light diffraction through the curved surface of the droplet. In the current study, an inverted confocal microscope was employed so that no specimen compression was required and whole animal imaging gave a relatively artefact-free image.

The importance of staining to reveal different structures is highlighted in this study, as the technique relies on the fluorescence of the specimen. The features of interest must either autofluoresce or be amenable to fluorescent staining to enable them to be visible using LSCM. For example, the sclerites of monogeneans are not autofluorescent and new staining techniques have been developed to allow them to be imaged successfully using LSCM (Galli et al. 2007, García-Vásquez et al. 2011). For taxonomic work, appropriate staining is important to ensure that all the anatomical features of the specimen are visible to avoid errors in the description. Michels & Büntzow (2010) demonstrated that Congo red is a very effective stain for viewing the exoskeleton of small crustaceans and cuticle of polychaetes using LSCM, as it selectively binds to chitin and collagen and fluoresces strongly around 561 nm. Their results showed that small structures were strongly fluorescent and that even after prolonged scan times and high laser intensities, specimen bleaching was negligible. Further experimentation with different staining techniques for copepods may improve results and allow finer features to be detectable. However, the

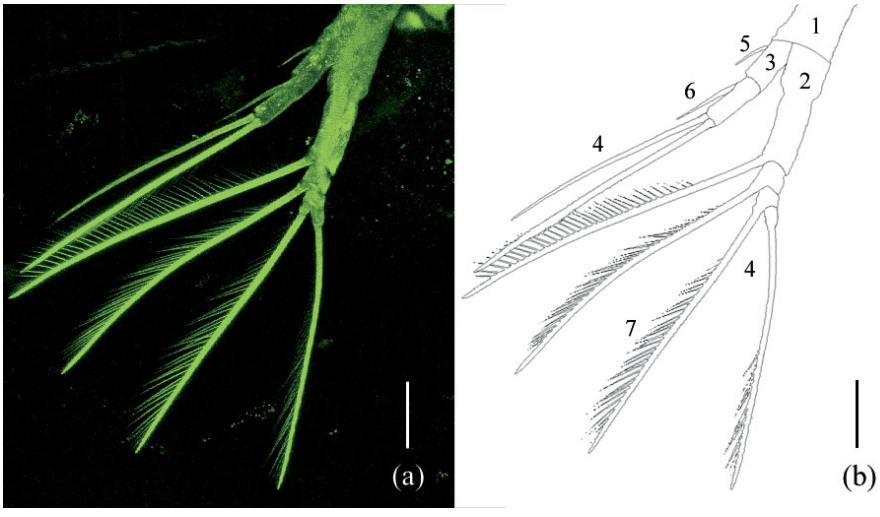

Fig. 6. *Lernaeocera branchialis* (L., 1767). Visualisation of fine features. (a) LSCM composite image of nauplius II mandible fixed in 2.5% glutaraldehyde and stained with Gomori's trichrome; (b) resultant line drawing showing how a contour filter can pick out fine hairs. Scale bars: 25 µm. Labels: 1, sympod; 2, exopodite; 3, endopodite; 4, plumose setae; 5, proximal setule; 6, distal setule; 7, fine hairs

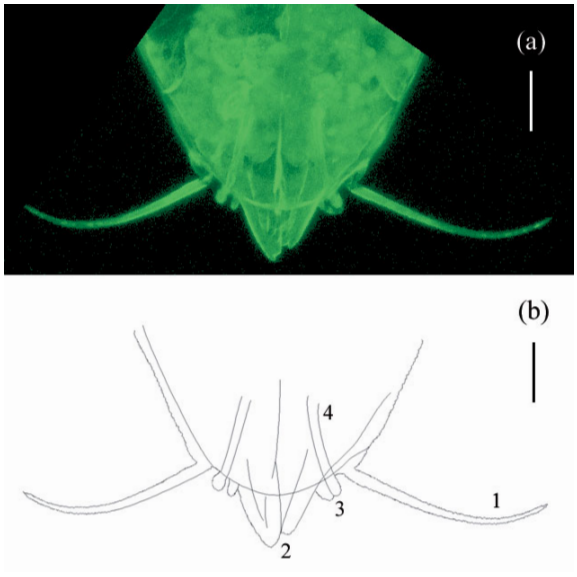

Fig. 7. *Lernaeocera branchialis* (L., 1767). Revealing internal structures. (a) LSCM composite image of nauplius II posterior fixed in 2.5% glutaraldehyde and stained with Gomori's trichrome; (b) resultant line drawing showing the protrusions at the terminal tip extending into the body. Scale bars: 25 µm. Labels: 1, balancers; 2, developing caudal rami; 3, protrusions; 4, internal structure of the protrusions

LSCM imaging process also produces a grey-scale pseudo light micrograph that may allow missing features to be added accurately. By using different stains, it is possible to view previously unseen structures, such as the small protrusions at the terminal tip of the nauplius II extending into the body when stained with Gomori's trichrome. Using phalloidin, which is a muscle stain, the musculature of specimens can be easily and accurately mapped, which may help identify the origin and function of appendages.

The only real limitation of using LSCM for taxonomy is the size of specimens that can be viewed. Large specimens may be too thick to fully image and, therefore, the technique is limited to smaller (~1 mm) specimens.

Digital taxonomy

The use of automated digital techniques to supplement taxonomic descriptions, promises to provide a new range of tools for systematic descriptions of copepods and other small invertebrates. Although this technique requires improvement, these early results demonstrate how taxonomic drawings might be quickly and effectively generated from LSCM images. Ramírez et al. (2007) commented that computer-generated taxonomic drawings require much skill and time to produce. However, with further investigation of different staining and contouring techniques, it should be possible with this technique to produce relatively accurate taxonomic drawings with minimum time and effort. Using these digital images, it would also be possible to utilise other automated techniques, such as size and shape descriptors, to further analyse specimens. Despite the increasing use of molecular systematics in recent years, such techniques still rely largely upon taxonomic descriptions defining key morphological characters for a species. Given the current rate of discovery of potential new species, it is essential that modern techniques are developed to assist the throughput of morphological taxonomy, which in turn provides the backbone for molecular systematics. Molecular techniques cannot replace morphological techniques, but rather the 2 should complement each other to in-crease our level of confidence in species descriptions and identifications (Hillis 1987, Monis 1999, Lipscomb et al. 2003, Will & Rubinoff 2004, Dayrat 2005).

One of the biggest hurdles of traditional taxonomy is the sharing and transport of material, especially species holotypes and paratypes, which are easily lost or damaged, or are simply not accessible in some countries. The unavailability of type specimens is a major impediment to rapid and accurate species identification, particularly for workers in the tropics (Balakrishnan 2005). With the renewed interest in natural history collections within the past 2 decades as a result of the biodiversity crisis (Alberch 1993, Dunn 2003), this problem is beginning to be addressed by the development of digital taxonomic databases accessible via the internet (Agosti & Johnson 2002, Bisby et al. 2002, Gewin 2002, Godfray 2002a,b, Mallet & Willmott 2003, Thacker 2003, Wheeler 2003, Harris et al. 2008, Smith et al. 2009). As with any other digital information, it would be simple and quick to share and disseminate digital images, including 2D image z-stacks and full 3D reconstructions of specimens viewable by anaglyph, traditional stereo-image pairs or through polarised viewing technologies, using email or other digital media, with no risk of damage or loss to individual specimens. Although these taxonomic images could not replace original holotype specimens, the use of LSCM data to generate 3D digital animations of specimens could be used as an alternative to holotypes and provide the cornerstone for digital taxonomic databases. Using these 'e-type' specimens (digital specimens stored and shared electronically),

it would be quick and easy to share this important taxonomic information with scientists around the world via online databases, allowing the original type specimens to be stored safely. The use of e-types to supplement holotypes requires further investigation, but depending on the technique employed to fix and mount the holotype, it may also be possible to image archived type specimens digitally using LSCM to create 3D e-types. For example, Gomori's trichrome is often used to stain monogeneans for light microscopy studies and the same specimens, which are held in helminth collections throughout the world, may be used for LSCM studies and 3D visualisation (Galli et al. 2007). One of the main difficulties of descriptive taxonomy is access to adequate reference collections (Gaston & O'Neill 2004), and the use of e-types will help to alleviate this problem by making virtual reference collections immediately available to anyone. By making these databases publicly available and allowing workers to deposit their own material, it will encourage collaboration between individual workers and research groups, allowing their work outputs to be integrated and increasing taxonomic productivity (Khuroo et al. 2007, Ramírez et al. 2007). There are, however, some caveats for creation of online resources. As is the case for holotypes and paratypes, there will, for instance, need to be some form of expert validation for e-types such that researchers using them can be assured that they are representative of the species in question and such databases will need to be curated appropriately to ensure that changes in nomenclature/taxonomic identity are appropriately reflected. These technologies may also enable, even without formal descriptions, large personal collections of specimens to be digitised and made publicly available online. It may also be possible to integrate e-type technology into automated species identification software packages that are under development (e.g. Dietrich & Pooley 1994, Arbuckle 2000, Gauld et al. 2000, Jonker et al. 2000, O'Neill et al. 2000, Arbuckle et al. 2001, Watson et al. 2004). In light of the decline in funding and available expertise for taxonomic research in recent years and the current biodiversity crisis (Gewin 2002, Gaston & O'Neill, 2004, Wheeler 2004, Wilson 2004, Khuroo et al. 2007), the ability to semi-automate descriptions using LSCM digital imaging can provide a range of tools to assist and improve morphology-based taxonomy. If these new tools and technologies are embraced by taxonomists and combined with traditional ones (Agnarsson & Kuntner 2007), they will help to strengthen and extend the capabilities of this essential discipline.

Acknowledgements. This project was funded by a NERC studentship awarded to A.J.B.

LITERATURE CITED

Agnarsson I, Kuntner M (2007) Taxonomy in a changing world: seeking solutions for a science in crisis. Syst Biol 56:531–539

Agosti D, Johnson NF (2002) Taxonomists need better access to published data. Nature 417:222

Alberch P (1993) Museums, collections and biodiversity inventories. Trends Ecol Evol 8:372–375

Arbuckle T (2000) Automatic identification of bee species from images of their wings. Proc 9th Int Workshop Systems, Signals and Image Processing. UMIST, Manchester, p 509–511

Arbuckle T, Schröder S, Steinhage V, Wittmann D (2001) Biodiversity informatics in action: identification and monitoring of bee species using ABIS. Proc 15th Int Symp Informatics for Environmental Protection. Metropolis, Zurich, p 425–430

Balakrishnan R (2005) Species concepts, species boundaries and species identification: A view from the tropics. Syst Biol 54:689–693

Bisby FA, Shimura J, Ruggiero M, Edwards J, Haeuser C (2002) Taxonomy, at the click of a mouse. Nature 418:367

Dayrat B (2005) Towards integrative taxonomy. Bot J Linn Soc 85:407–415

Dietrich CH, Pooley CD (1994) Automated identification of leafhoppers (Homoptera: Cicadellidae: *Draeculacephala* Ball). Ann Entomol Soc Am 87:412–423

Dunn CP (2003) Keeping taxonomy based in morphology. Trends Ecol Evol 18:270–271

Galli P, Strona G, Villa AM, Benzoni F, Stefani F, Doglia SM, Kritsky DC (2006) Three-dimensional imaging of monogenoidean sclerites by laser scanning confocal fluorescence microscopy. J Parasitol 92:395–399

Galli P, Strona G, Villa AM, Benzoni F, Stefani F, Doglia SM, Kritsky DC (2007) Two-dimensional versus three-dimensional morphometry of monogenoidean sclerites. Int J Parasitol 37:449–456

García-Vásquez A, Shinn AP, Bron JE (2011) Development of a light microscopy stain for the sclerites of *Gyrodactylus* von Nordmann, 1832 (Monogenea) and related genera. Parasitol Res doi: 10.1007/s00436-011-2675-y

Gaston KJ, O'Neill MA (2004) Automated species identification: Why not? Phil Trans R Soc Lond B 359:655–667

Gauld ID, O'Neill MA, Gaston KJ (2000) Driving Miss DAISY: the performance of an automated insect identification system. In: Austin AD, Dowton M (eds) Hymenoptera: evolution, biodiversity and biological control. CSIRO, Collingwood, p 303–312

Gewin V (2002) All living things, online. Nature 418:362–363

Godfray HCJ (2002a) Challenges for taxonomy. Nature 417: 17–19

Godfray HCJ (2002b) Towards taxonomy's 'glorious revolution'. Nature 420:461

Gomori G (1950) A rapid one-step trichrome stain. Am J Clin Pathol 20:661–664

Harris PD, Shinn AP, Cable J, Bakke TA, Bron J (2008) Gyrodb: gyrodactylid monogeneans on the web. Trends Parasitol 24:109–111

Hillis DM (1987) Molecular versus morphological approaches to systematics. Annu Rev Ecol Syst 18:23–42

Jonker R, Groben R, Tarran G, Medlin L and others (2000) Automated identification and characterisation of micro-

bial populations using flow cytometry: the AIMS project. Sci Mar 64:225–234

Khuroo AA, Dar GH, Khan ZS, Malik AH (2007) Exploring an inherent interface between taxonomy and biodiversity: current problems and future challenges. J Nat Conserv 15:256–261

Klaus AV, Kulasekera VL, Schawaroch V (2003) Three-dimensional visualization of insect morphology using confocal laser scanning microscopy. J Microsc 212:107–121

Lipscomb D, Platnick N, Wheeler Q (2003) The intellectual content of taxonomy: a comment on DNA taxonomy. Trends Ecol Evol 18:65–66

Mallet J, Willmott K (2003) Taxonomy: Renaissance or Tower of Babel? Trends Ecol Evol 18:57–59

Michels J, Büntzow M (2010) Assessment of Congo red as a fluorescence marker for the exoskeleton of small crustaceans and the cuticle of polychaestes. J Microsc 238: 95–101

Monis PT (1999) The importance of systematics in parasitological research. Int J Parasitol 29:381–388

O'Neill MA, Gauld ID, Gaston KJ, Weeks PJD (2000) DAISY: an automated invertebrate identification system using holistic vision techniques. In: Chesmore D, Yorke L, Bridge P, Gallagher S (eds) Proc Inaug Meet BioNET-INTERNATIONAL Group for Computer-Aided Taxonomy (BIGCAT). BioNET-INTERNATIONAL Technical Secretariat, Egham, p 13–22

Ramírez MJ, Coddington JA, Maddison WP, Midford PE and others (2007) Linking of digital images to phylogenetic data matrices using a morphological ontology. Syst Biol 56:283–294

Smith VS, Rycroft SD, Harman KT, Scott B, Roberts D (2009) Scratchpads: a data-publishing framework to build, share and manage information on the diversity of life. BMC Bioinformatics 10(Suppl 14):S6

Thacker PD (2003) Morphology: the shape of things to come. Bioscience 53:544–549

Watson AT, O'Neill MA, Kitching IJ (2004) Automated identification of live moths (Macrolepidoptera) using Digital Automated Identification System (DAISY). Syst Biodivers 1:287–300

Wheeler QD (2003) Transforming taxonomy. Systematist 22: 3–5

Wheeler QD (2004) Taxonomic triage and the poverty of phylogeny. Phil Trans R Soc Lond B 359:571–583

Will KW, Rubinoff D (2004) Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics 20:47–55

Wilson EO (2004) Taxonomy as a fundamental discipline. Phil Trans R Soc Lond B 359:739

Editorial responsibility: Söhnke Johnsen, Durham, North Carolina, USA

Submitted: March 11, 2011; Accepted: October 25, 2011
Proofs received from author(s): December 31, 2011