



The use of hand-sanitiser gel facilitates combined morphological and genetic analysis of shelled pteropods

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Abstract

There is a lack of standardised imaging methods for marine zooplankton due to the difficulty of manipulating small and often fragile specimens. Yet, standardised 2D photographs and 3D scans provide important morphological information to accompany DNA-barcoded specimens for reference databases such as the Barcode of Life Data System (BOLD). Shelled pteropods are considered as bio-indicators to study impacts of ocean acidification, and thus, it is especially important to obtain high-quality records of their fragile aragonitic shells. We used alcohol-based hand sanitiser gel as a medium for photographing pteropods of the genus *Limacina* prior to micro-CT scanning and destructive DNA analysis. The high viscosity and transparency of the hand sanitiser enabled easy handling of the specimens so that they could be positioned in a standardised orientation and photographed with a stacking microscope. The high-quality photographs provide a record of morphology and allow for subsequent geometric morphometric analyses. This method did not impact the downstream micro-CT and molecular analyses of the same specimens and resulted in publicly available 2D and 3D digital vouchers as well as ten reference DNA barcodes (partial Cytochrome Oxidase I gene sequences). While alcohol-based hand sanitiser entered our daily lives due to a distressing pandemic, we could make use of it as a cheap and easily available resource to make high quality voucher photographs of shelled pteropods. Digital vouchers serve as a record of their morphology for further taxonomic analyses and facilitate studies assessing shell growth and impacts of ocean acidification.

Keywords Integrative taxonomy · *Limacina* · Digital vouchers · DNA barcoding · Marine zooplankton

Introduction

Shelled pteropods are holoplanktonic gastropods that are a common subject of global change research, due to their important ecological and biogeochemical roles and susceptibility to

ocean acidification. Shelled pteropods have been regarded as bio-indicators of ocean acidification, because their thin aragonitic shell is sensitive to dissolution under acidified conditions (Orr et al. 2005; Bednaršek et al. 2017). Pteropods contribute to the oceanic carbon flux by producing biomass and sequestering carbon through high phytoplankton grazing (Hunt et al. 2008) and large downward fluxes of faecal pellets (Manno et al. 2010), mucus nets (Noji et al. 1997; Conley et al. 2018), and shells (Tsurumi et al. 2005; Fabry et al. 2009). Pteropods are also prey for heteropods (Böer et al. 2005), amphipods (Bernard 2006), cephalopods (Hanlon and Messenger 1998), and, in polar systems, fishes, seabirds (Hunt et al. 2008), and marine mammals (Lalli and Gilmer 1989). Their species diversity and population structure are commonly assessed with DNA barcodes (e.g. Hunt et al. 2010; Jennings et al. 2010; Burrige et al. 2017; Kohnert et al. 2020; Choo et al. 2021), which contributes to a global zooplankton DNA barcoding reference database.

There is an urgent need for more high-quality voucher photographs of specimens to complement DNA barcode reference databases of marine zooplankton (Bucklin et al.

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2021). This allows for the morphological identification of specimens, and validation of their DNA barcodes (Bucklin et al. 2016; Laakmann et al. 2020). The preservation of morphological characters of the specimen, in addition to sequencing of DNA barcodes, and collection of associated georeferencing, environmental, and ecological metadata, are important components of an integrative taxonomy pipeline (Padial et al. 2010). With the aid of these multiple sources of information, we can successfully assess species boundaries within the zooplankton assemblage (Hirai et al. 2015; Bode et al. 2017; BurrIDGE et al. 2019), and in some cases, resolve (pseudo-)cryptic species identified through DNA barcodes with more thorough inspection of their morphology (e.g. BurrIDGE et al. 2015; Wall-Palmer et al. 2018). While non-destructive DNA extraction is possible in some zooplankton taxa by subsampling (e.g. Choquet et al. 2018), or by leaching DNA without damage to chitinous exoskeletons or calcite shells (e.g. Cornils 2015; Weiner et al. 2016), we have had no success in preserving the highly soluble aragonitic shells of pteropods using different DNA extraction protocols. In such cases, high-quality voucher photographs play a critical role as the only remaining record of the morphological traits of the DNA barcoded specimen.

There is a lack of methodological detail as to how zooplankton can be prepared for taking a high-quality stacking photograph. Ideally, these specimens should be positioned in a standardised orientation to facilitate measurements of body parts, or to highlight a particular aspect of their anatomy. In coiled gastropods especially, the positioning of the specimen is important to standardise the turn and tilt of the shell (Callomon 2019), which directly impacts the suitability of the photographs in 2D geometric morphometrics analyses. Known methods for positioning small invertebrates for photography vary from malleable tack for dry specimens to glass slides, wax cradles, and stainless steel nuts for wet specimens (Geiger et al. 2007). Micro-computed tomography (micro-CT) scanning is a method where specimens do not need to be placed in a standardised orientation because 3D reconstructions of their shell morphology are obtained (Shimizu et al. 2018, 2021), but it is expensive and time-consuming to have 3D scans for all specimens that are included in a DNA barcoding pipeline.

As part of our integrative taxonomy pipeline for planktonic gastropods, we needed to find a reproducible way to image specimens in a standardised orientation before destructive DNA extraction. Alcohol-based hand sanitiser gel became increasingly more common since the start of the COVID-19 pandemic as a convenient replacement of soap and water to maintain hand hygiene. Because of the availability of alcohol-based hand gel, we were inspired to include it as part of our integrative taxonomy workflow to facilitate the photography of small pteropods. Alcohol-based hand sanitiser has been commonly used in positioning and stabilising spiders (e.g. Valdez-Mondragón 2010; Bilton 2018; LeMay and Agnarsson 2020)

and other macroinvertebrates (<http://www.gigamacro.com/blog/wet-specimen-holder-macroinvertebrates>) for photography; however, it had not been applied to planktonic gastropods or other marine zooplankton. Here, we explored the use of alcohol-based hand sanitiser to obtain high quality 2D images of shelled pteropods, and subsequently 3D micro-CT scans prior to destructive DNA analyses.

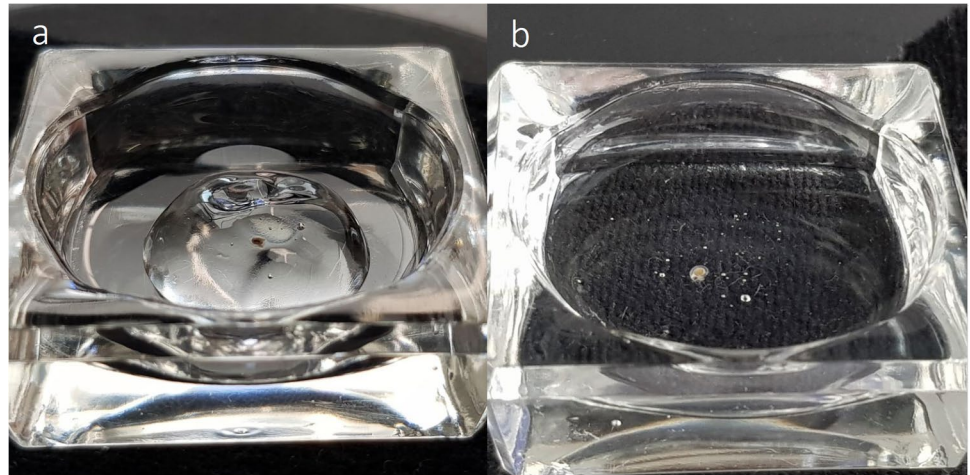
Material and methods

We used alcohol-based hand sanitiser in the photography of ten shelled pteropods belonging to the *Limacina* genus, representing five nominal species: *L. helicina* s.l., *L. retro-versa* s.l., *L. bulimoides*, *L. trochiformis*, and *L. lesueurii*. Previously, fine black sand was used to position pteropods in a standardised orientation for photography (e.g. BurrIDGE et al. 2017; Choo et al. 2020), although the noise in the background of images meant that they were unsuitable for downstream image recognition or machine learning applications. Here, alcohol-based hand gel was used as a positioning medium for photographing the specimen in a standardised apertural orientation. The store-bought hand sanitiser we used (“Dr. Original” brand) contained the following ingredients: denatured alcohol, water, polyacrylamide, C13-14 Isoparaffin, and Laureth-7.

Under the microscope, we prepared the setup for imaging, which consisted of a watch glass with a small volume of hand sanitiser, atop on a black velvet cloth which provided a dark background (Fig. 1a). Each specimen was gently placed on the blob of hand sanitiser in a watch glass. The specimen was then manipulated carefully into a standard orientation with a thin brush, to ensure the shell axis was parallel to the plane of the camera (Fig. 1a). The specimen and hand sanitiser were then covered with a thin layer of 96% ethanol to reduce glare (Fig. 1b). Subsequently, the shells were photographed using a Zeiss V20 stacking stereomicroscope with Axiovision software (Zeiss, Germany). Once photographed, each specimen was rinsed in 96% ethanol to wash off remaining gel and stored in 96% ethanol at -20°C .

After the photograph was obtained for each specimen, a subset of five individuals was selected for micro-CT scanning. Specimens were prepared and scanned according to the protocol described in (Mekkes et al. 2021). We also obtained the mitochondrial cytochrome oxidase subunit I (COI) barcode of the 10 individuals. DNA was extracted and barcoded according to the protocol in (Choo et al. 2021). The shells were washed in 96% ethanol before micro-CT scanning and in MilliQ water before being placed in lysis buffer for DNA extraction to prevent possible inhibition of the polymerase chain reaction (PCR) due to any remaining (denatured) alcohol. Sequences were checked and edited in Geneious Prime

Fig. 1 Imaging setup for *Limacina* specimens. **a** A watch glass with hand sanitiser added was placed on a sheet on black velvet under the microscope. The specimen was placed within the hand sanitiser and then manipulated into position. **b** A small volume of 96% ethanol was added to completely cover the hand sanitiser.



2021.1.1 (<https://www.geneious.com>) and submitted to the BOLD database (Ratnasingham and Hebert 2007, 2013).

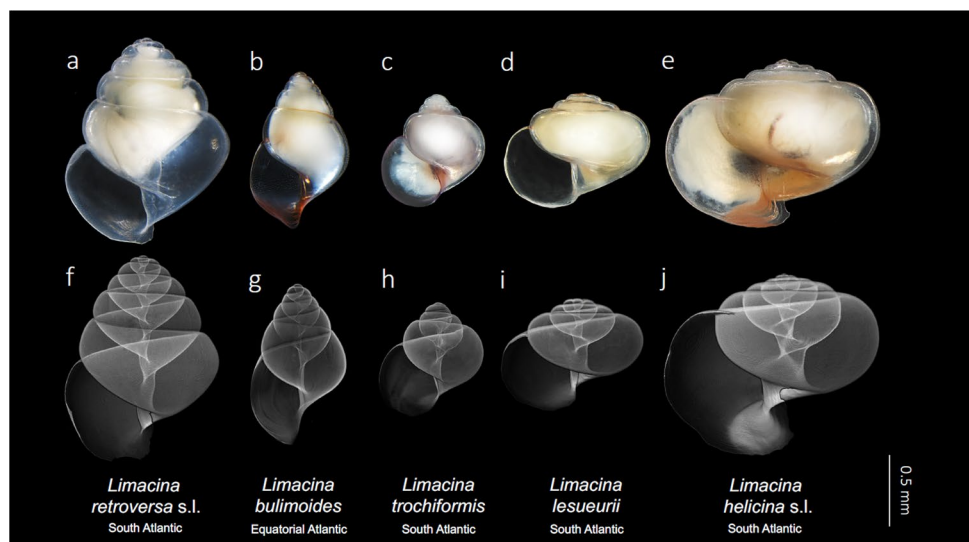
Results and discussion

Alcohol-based hand sanitiser facilitated the imaging process of ethanol-preserved shelled pteropods and did not interfere with their downstream micro-CT scanning, DNA extraction, and molecular analyses. We experienced that orienting the specimens for stacking photography was efficient with alcohol gel and the transparent shells contrasted well against the clean dark background provided by the black velvet cloth. These high-quality images can be used for publication and geometric morphometric analyses and were included as digital vouchers in the BOLD reference database with minimal editing needed (Fig. 2).

The DNA extraction and COI barcoding of the 10 specimens were successful, including for those five

specimens (one for each species) that had the additional step of micro-CT scanning prior to DNA extraction. This adds further evidence to initial exploratory experiments (Hall et al. 2014) that DNA barcoding is compatible with micro-CT scanning and barcodes can be reliably amplified despite the X-ray radiation from the scanning. The DNA vouchers of these specimens are stored and accessible through Naturalis Biodiversity Center in Leiden, the Netherlands (Museum accessions: RMNH.MOL.347126, 347129, 347279-280, 347361, 347363, 347367-68, 347375, 347382), and their 2D photographs and COI barcodes were submitted to BOLD (BOLD accessions: LPCOI001-21-LPCOI010-21). Their micro-CT reconstructions were uploaded onto MorphoSource with digital object identifiers (DOIs) and can be accessed via <https://www.morphosource.org/projects/000539891> with their respective DOIs. We note that two of the taxa *L. retroversa* s.l. and *L. helicina* s.l. require taxonomic revision (WoRMS Editorial Board 2021).

Fig. 2 Stacking photographs (top row) and micro-CT 3D reconstructions (bottom row) of one specimen from each of the five nominal *Limacina* species. (a, f) *L. retroversa* s.l., (b, g) *L. bulimoides*, (c, h) *L. trochiformis*, (d, i) *L. lesueurii*, and (e, j) *L. helicina* s.l. The stacking photographs and micro-CT reconstructions for each species are of the same individual, and species name and collection locality are given below each individual. *Sensu lato* (s.l.) is included for the species requiring taxonomic revision.



Our method of photography with alcohol-based hand sanitiser allowed for easy manipulation of small pteropods while providing enough viscosity to stabilise specimens during stacking photography. To support heavier specimens, we cooled the gel in the fridge to increase its viscosity. Different store-bought hand sanitiser brands have varying ingredient compositions that affect their viscosity and transparency (Berardi et al. 2020); therefore, this may require some testing for their suitability before use. Alcohol-based hand sanitisers also vary in terms of their alcohol concentrations, between the range of 60 and 95% (Boyce and Pittet 2002; Edmonds et al. 2012), though we expect that the initial fixation quality of the specimen (ideally in >95% ethanol) matters more than the ethanol concentration of the alcohol gel when it comes to subsequent DNA extraction and PCR (Stein et al. 2013). Alternative media used in positioning specimens for photo-documentation include glycerol, glycerine-gelatine jelly, and water-based lubricating jelly (Evitt 1984; Sasso Porto et al. 2016) although these do not contain ethanol and may affect downstream DNA analyses.

We suggest that the use of alcohol gel for photography can be integrated in the digitisation and barcoding pipelines of other marine zooplankton taxa for reference databases. High-quality voucher photographs of specimens are important to link genetic information with phenotypic characters through high-quality photographs and 3D reconstructions, especially if specimens have to be destroyed for DNA extraction (Bucklin et al. 2021). This is a useful approach for other calcifying zooplankton such as foraminifers and other pelagic gastropods, as well as for chaetognaths and polychaetes (personal observation). The increased flexibility in positioning specimens could also allow for better observation of diagnostic traits and more accurate measurements of appendages in e.g., crustaceans, and could facilitate re-assembly of fragile species that fall apart upon collection as for gelatinous groups such as siphonophores. Furthermore, the transparency of the gel allows to switch between different colours of background by simply using a different coloured cloth under the watchglass. While a dark background is commonly used in publications and typically allows for better thresholding (e.g. for machine learning or image recognition applications), a light background is better suited for analyses of colour variation, which may be indicative of species boundaries in e.g. *Limacina bulimoides* (Choo et al. 2023).

While alcohol-based hand sanitiser entered our daily lives due to a distressing pandemic, we learned that we could make use of it as a cheap and easily available resource as part of an integrative taxonomy pipeline for shelled pteropods. The ease of positioning specimens in a standard orientation also facilitates the challenging photography of younger life stages, such as veliger larvae in gastropods and nauplii in crustaceans, prior to DNA extraction. This is necessary

to provide a complete overview of morphology across the life history of the species, facilitate species identifications, and resolve (pseudo-) cryptic species complexes. We hope that the described methodology can increase the efficiency of collecting morphological information and encourage the inclusion of high quality 2D and 3D digital vouchers in DNA reference databases for marine zooplankton.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval No animal testing was performed during this study.

Sampling and field studies All necessary permits for sampling have been obtained by the authors from the competent authorities.

Data availability The data underlying this article are available in BOLD at <http://www.boldsystems.org/>, and can be accessed with BOLD Process IDs LPCOI001-21 to LPCOI010-21. The DNA vouchers (RMNH.MOL.347126, 347129, 347279-280, 347361, 347363, 347367-68, 347375, 347382) are accessioned at Naturalis Biodiversity Center, the Netherlands, and are available upon request. The 3D scans are archived on MorphoSource and can be accessed at <https://www.morphosource.org/projects/000539891> or via the following DOIs: doi:<https://doi.org/10.17602/M2/M486506>, doi:<https://doi.org/10.17602/M2/M486512>, doi:<https://doi.org/10.17602/M2/M486521>, doi:<https://doi.org/10.17602/M2/M486529>, doi:<https://doi.org/10.17602/M2/M495278>, doi:<https://doi.org/10.17602/M2/M495286>, doi:<https://doi.org/10.17602/M2/M495296>, doi:<https://doi.org/10.17602/M2/M495315>, doi:<https://doi.org/10.17602/M2/M495327>, doi:<https://doi.org/10.17602/M2/M495345>.

Author contribution KTCAP, GS, and LQC designed the research. KTCAP and LQC contributed samples used for the study. GS performed sample processing and analyses. LQC wrote the manuscript. All authors reviewed and approved of the manuscript.

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